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LABORATORY PROCEDURES FOR THE DIAGNOSIS OF INTESTINAL PARASITES

Dorothy M. Melvin, et al

National Communicable Disease Center
Atlanta, Georgia

1969

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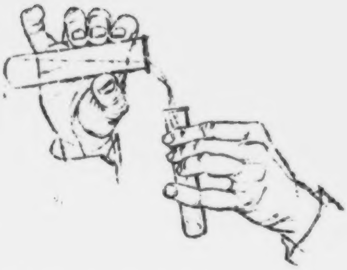
LABORATORY PROCEDURES



FOR

THE DIAGNOSIS OF INTESTINAL PARASITES

Dorothy M. Melvin, Ph.D.
and
Marion M. Brooke, Sc.D.

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
Health Services and Mental Health Administration
NATIONAL COMMUNICABLE DISEASE CENTER
Laboratory Division
Laboratory Consultation and Development Section
Atlanta, Georgia 30333

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PREFACE

This manual is based on material developed for courses in the Laboratory Diagnosis of Parasitic Diseases which the National Communicable Disease Center (NCDC) has presented since 1945. The authors have been associated with these courses since their beginning and as a consequence have had the opportunity during 24 years to evaluate a great deal of material in the preparation of laboratory manuals and brochures for student use. The present manual contains information on practical procedures for performing parasitologic examinations in public health and medical laboratories. It includes information on collecting and processing specimens, methods of examination, and establishing reliable routine laboratory regimens for different types of laboratories. It does not contain morphologic information on the diagnostic stages of the parasites. The diagnostic stages of intestinal helminths and protozoa have been described by the authors in another publication: "Morphology of the Diagnostic Stages of Intestinal Parasites of Man" (Brooke and Melvin, in press), which also will be available from the Government Printing Office.

The manual is divided into two sections: (1) a text section which describes and evaluates the available diagnostic procedures and methods, and (2) a techniques section which gives step-by-step directions for diagnostic procedures, formulas for solutions, and methods for preparing and examining specimens. The techniques section also contains information on microscopy and on the calibration of ocular micrometers.

Some of the material in this manual has been taken from "Amebiasis: Methods in Laboratory Diagnosis" by M. M. Brooke (out of print) and the self-instructional lesson prepared by NCDC, "Amebiasis: Laboratory Diagnosis: Part III: Laboratory Procedures." The latter may be obtained from the Superintendent of Documents, U. S. Government Printing Office, Washington, D. C., 20402.

The authors acknowledge the contributions of numerous associates over the years and thank the various people who have assisted in the preparation of the manual, particularly Miss Edwina B. Davis, technical-writer-editor, who edited the material and Mr. John H. Krickle, Education Specialist, who helped in developing the format of the manual.

Dorothy M. Melvin, Ph.D.
Chief, Parasitology Training Unit

M. M. Brooke, Sc.D.
Chief, Laboratory Consultation and Development Section

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INTRODUCTION

Accurate clinical diagnosis of intestinal parasitic disease is difficult and laboratory confirmation is usually necessary. Demonstrating the diagnostic stage or stages of the parasite by directly examining specimens is the most reliable method of establishing a diagnosis of most parasitic infections, although indirect (serologic) methods are available for a few in which the organism is not readily demonstrated, including trichinosis, echinococcosis, extra-intestinal amebiasis and chronic schistosomiasis.

This manual is concerned with direct laboratory procedures used in recovery and identification of intestinal parasites and related species (atrial, bladder, and lung parasites) and with the collection of satisfactory specimens. Competent laboratory work is dependent on several factors: 1) personnel trained in examining specimens and accurately identifying organisms, 2) adequate laboratory facilities, including a good microscope, and 3) satisfactory specimens. Any laboratory procedure used for directly demonstrating parasites is only as reliable as the microscopist who examines the specimen; therefore, all persons attempting to make parasitologic examinations should have intensive training and supervised experience.

The primary purpose of this manual is to present selected methods which can be used routinely in laboratories performing parasitologic examinations and which, when performed properly, are reliable and thorough. Many excellent techniques available for laboratory diagnosis of parasitic diseases, however, are not included.

SPECIMEN COLLECTION

The usual diagnostic stages of intestinal parasites are helminth eggs and larvae and protozoan trophozoites and cysts. Specimens obtained for diagnosis should be collected and handled so that these stages, when present, will reach the laboratory in an identifiable condition. In fact, reliable diagnoses of parasitic diseases begins with satisfactory materials, and the importance of properly collected specimens cannot be overemphasized. Inadequate samples and old or poorly preserved materials are usually of little or no value in establishing a diagnosis, and they may lead to erroneous conclusions.

The most common type of body material submitted for parasitologic examination is feces, but other materials, such as sputum, urine, aspirates, tissue scrapings, and specimens obtained by biopsy may be submitted in certain cases.

FECAL SPECIMENS

NORMALLY PASSED—To obtain satisfactory material for examination, adequate instructions and proper containers for collecting fecal specimens must be provided. Persons who have little or no knowledge of the proper procedures for submitting samples to the laboratory often make the collections; therefore, complete and explicit directions should be given to the person responsible for obtaining the specimen. Printed instructions with line drawings to illustrate certain steps are usually included in the collection kits the laboratory supplies. In hospitals or clinics, specimens may be collected in bedpans if the feces are not contaminated with urine.

Information identifying the patient and the date and hour of passage should be recorded on the carton or should accompany the specimen. The time of passage is especially important, since trophozoites soon die outside the body and old specimens may be erroneously reported as negative. A suggested identification label for specimen containers is as follows:

Name________________________________
____
Age________ Male ( )
Female ( )
Date ______________________________ and
hour________ AM PM stool passed

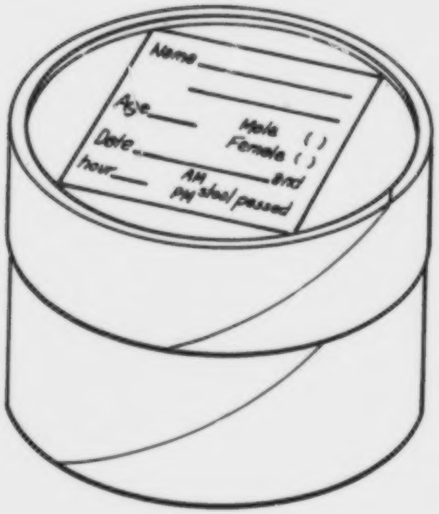
PURGED—In some cases, purged specimens may be desirable or necessary. Andrews (1934), Magath (1934), Aliona and Fadell (1959) and others have shown that purgation increases the possibility of finding organisms in a single specimen. In cases of protozoan infections, however, trophozoites predominate in purged specimens, and species identification of trophozoites is usually less reliable than identification based on cysts (Bijlmer, 1948; Brooke *et al.*, 1953; Stamm, 1957; and Goldman, 1964). Saline cathartics, such as sodium sulfate or buffered phospho-soda, should be used for purgation. Mineral oil, bismuth, or magnesia compounds are unsatisfactory. Oil globules interfere with examination, and crystalline debris from bismuth and magnesia compounds may obscure the organisms or affect the appearance of trophozoites.

Fecal specimens should be passed into clean, dry containers or on clean paper and transferred to some type of container suitable for transportation to the laboratory. Feces deposited on the soil are not satisfactory, since free-living larvae and other contaminants from the soil may cause errors in diagnosis. Feces obtained from toilet bowls are equally unsatisfactory. Not only is there danger of coprozoic organisms contaminating the specimens, but, also, water will destroy trophozoites that might be present. In addition, feces mixed with urine are not acceptable since, like water, urine will destroy trophozoites.

Specimens collected in very cold regions should not be allowed to freeze, since freezing and thawing may destroy protozoan cysts and trophozoites (Goldman and Johnson, 1950; Chang, 1955).

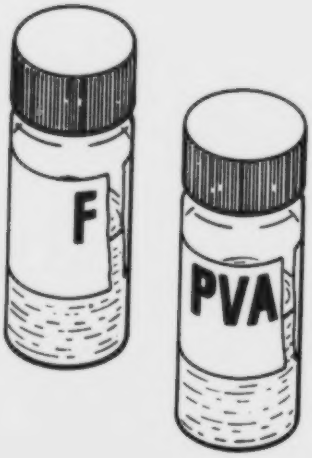
Certain drugs and compounds will render the stool specimens unsatisfactory for examination or for detection of organisms. Among these are antidiarrheal compounds, antibiotics, antacids, bismuth, and barium (Juniper, 1962). Specimens for parasitologic diagnoses should be obtained before these compounds are used, otherwise collection must be delayed until the effects have passed. Specimens

Figure 1.
Containers for Collection of Fecal Specimens


Name
Age
Male ( )
Female ( )
Date
hour
AM
PM
and
stool passed

Carton for Unpreserved
Specimens Submitted
Immediately to the Laboratory

Two Vials Containing Formalin
and PVA-Fixative Solutions
for Preserving Specimens
which ARE NOT Sent
Immediately to the Laboratory



should not be collected for 7 to 10 days after barium or bismuth have been given, since crystals and particles of these compounds in the feces will interfere with examination. Antibiotics often cause a temporary decrease or absence of organisms in the stools and reliable diagnoses may not be possible for 2 to 3 weeks or more.

To permit an adequate gross and microscopic examination, whenever possible, the whole fecal passage should be submitted. A waxed half-pint carton with an overlapping lid is recommended for the routine collection of specimens (Figure 1). This type of carton can be defecated into directly, does not leak, and permits labelling on the box. Larger sizes are also available.

The patient receiving catharsis should be directed to collect all bowel movements in separate boxes numbered in series and to deliver them immediately to the laboratory. Post-cathartic specimens should be examined for eggs, larvae, and cysts as well as for trophozoites. If the examination may be delayed, a portion of the specimen should be mixed with polyvinyl alcohol fixative (PVA-fixative) preferably upon collection or as soon afterwards as possible. This practice may be advisable even in clinics and hospitals where, theoretically, the specimens are taken directly to the laboratory and examined, but where, actually, considerable delay may occur.

MULTIPLE SPECIMENS—Because of the intermittent passage of certain parasites from the host and the limitations of available diagnostic techniques, the possibility of finding organisms is increased by examining multiple specimens (Andrews, 1934; Sawitz and Faust, 1942; Wykoff and Ritchie, 1952; Swartzwelder, 1952; Alicna and Fadell, 1959). A single, normally passed specimen reveals only about one-third to one-half of the species present. Thus, a physician receiving a report of "no parasites found" on a single specimen should realize that this does not exclude the possibility of his patient being infected. In general, nematode species, such as *Ascaris lumbricoides*, hookworm, and *Trichuris trichiura*, shed eggs more or less constantly and may be detected daily in feces. Other parasite species, however, especially the protozoa, are passed irregularly. The number of cysts of *Entamoeba histolytica* passed in the stools fluctuates from day to day with cyclical peaks occurring 8 to 10 days apart (Tsuchiya, 1932; Kershaw, 1946; Stamm, 1957). Lincicome (1942) found that in monkeys, maximal numbers of *E. histolytica* and *Entamoeba coli* were shed at approximately weekly intervals; Marsden (1946) maintained that they are passed at random intervals of only a few days duration. Egg production in certain of the helminth infections, particularly the schistosomes and *Diphyllobothrium latum*, is also irregular. Proglottids of *Taenia* species may be passed at intervals. Thus, collecting specimens at 2- to 3-day intervals seems preferable to obtaining them on successive days.

Workers do not agree on the number of specimens that should be examined to rule out infections. If time permits, however, at least 2 or 3 normally passed stool specimens, preferably spaced several days apart, should be examined before one resorts to catharsis or sigmoidoscopy. Normally passed specimens are more likely to contain cysts which, as already stated, can be identified with greater reliability than trophozoites. Since organisms may be rare in passages following purgation for a week or more, normally passed specimens should be collected before the purge is given.

STORAGE, PRESERVATION, AND SHIPMENT

Unpreserved Specimens—Since protozoan trophozoites do not multiply or encyst outside the body, they are destined to degenerate and die. Unpreserved specimens, therefore, should be examined as soon as possible. Ideally, specimens should be passed in close proximity to the laboratory so that they can be examined shortly after passage. If feasible, the feces should be examined while warm, especially if protozoan infections are suspected and the specimen is soft or diarrheic. If the examination is delayed, however, the fecal specimen should not be kept artificially warm. Elevated temperatures hasten the death of the organisms, particularly of amebae trophozoites (Tsuchiya, 1945; Gurevitch and Delightish, 1947). The survival times for *E. histolytica* trophozoites (Tsuchyia, 1945) and cysts (Chang and Fair, 1941; Simitch *et al.*, 1945) follow. Although helminth eggs and larvae are more resistant to environmental changes than the protozoan cysts and trophozoites, development or hatching which could complicate diagnosis may occur. In general, therefore, if a fresh specimen cannot be examined immediately, keep it at room temperature or in the refrigerator for the next few hours.

SURVIVAL TIMES FOR *ENTAMOEBA HISTOLYTICA*

Stage	Medium	TEMPERATURE		
		37°C	20-25°C	5°C
Trophozoites	Feces	2-5 hours	6-16 hours	48-96 hours
Cysts	Feces	1-2 days	3- 4 days	14-40 days
	Water	2 days	10 days	42 days

Preserved Specimens—Many diagnostic laboratories receive specimens through the mails. If these are delayed in reaching the laboratory and are not preserved properly, any organisms present, particularly protozoa, may be unrecognizable or completely disintegrated by the time the specimen is examined. Adequate and proper preservation is, therefore, essential. In addition, the shipping containers must meet postal requirements for mailing laboratory materials. These are described in the paragraph entitled "Mailing Specimens."

For preserving eggs, larvae, and cysts, 5 to 10 percent formalin is satisfactory. To insure complete preservation use about 3 volumes of formalin to 1 volume of feces and thoroughly mix the specimen. In 1949, Brooke and Goldman described a method of preserving intestinal protozoa in vials of PVA-fixative, a solution containing a water soluble resin and Schaudinn's fixative. The formula for preparing this fixative and directions for its use are described in the section on Techniques. As with formalin, about 3 volumes of PVA-fixative should be added to 1 volume of feces and the specimen thoroughly mixed. After being preserved in PVA-fixative, any trophozoites present will remain suitable for staining and identification for several months. Different investigators have demonstrated the effectiveness of PVA-fixative in preserving the intestinal protozoa, particularly the trophozoite stage (Young *et al.*, 1951; Goldman and Brooke, 1953; Regonesi *et al.*, 1954; Buonomini and Braccini, 1954; Artigas, 1955; Brooke, 1960; Brooke *et al.*, 1955, 1956, 1963a, 1963b; Myers, *et al.*, 1959; Melvin and Brooke, 1962).

Brooke and Goldman (1949) have advocated a two-vial method of shipping stool specimens which enables the laboratory to recover all diagnostic stages of the intestinal parasites (Figure 1). One vial of the kit contains PVA-fixative and the other contains 5 to 10 percent formalin, or if the specimen can be received within 1 or 2 days, it is left empty. Screw-cap vials of 20 to 30 ml capacity are recommended. In the United States, a number of State public health laboratories have provided such shipment kits with good results (Kelley, 1952; Harper *et al.*, 1957). PVA-fixative is stable and may be kept for long periods in tightly stoppered bottles, but, when it is dispensed in small vials, it may harden or jell after a time and become unsuitable for use. Therefore, to be sure that the fixative in the collection kit is in good condition, an expiration date should be indicated on the vial. Two months is a practical time limit for routine purposes.

Another satisfactory preservative for stool specimens is the merthiolate-iodine-formaldehyde (MIF) solution (Sapero and Lawless, 1953) which preserves trophozoites as well as cysts, eggs, and larvae. MIF solution has been recommended for survey work when only one procedure, the MIF concentration (Blagg *et al.*, 1955), is performed. (See section on Techniques.) This solution can be substituted for formalin in the two-vial collection method. However, MIF may complicate the collection too much for routine use in a diagnostic laboratory. In using MIF as a preservative, the iodine solution must be added to the merthiolate-formalin solution just before the specimen is collected. This is not a complicated manipulation, but many patients have difficulty following the simplest directions without individual instructions. Furthermore, the iodine solution deteriorates within a few weeks but formalin solutions last indefinitely. Many of the cysts and some trophozoites can be identified in wet mount preparations (MIF and others), but identification of trophozoites should be confirmed in permanently stained films. If only MIF preserved specimens are collected, permanent stains for critical study and storage of positive findings cannot be prepared. A vial of PVA-fixative should be included in the collection kit so that stained slides can be prepared.

Burrows (1967a) has recently developed a phenol-alcohol-formaldehyde fixative (PAF-fixative) for trophozoites, cysts, eggs and larvae which may be used for routine purposes. The formula and procedure for use are given in the section on Techniques. In the laboratory, the material can be examined by direct wet mounts. A drop of thionin stain should be used to bring out structural details of protozoan trophozoites and cysts. Nuclear details are particularly well-defined. The specimens can also be concentrated by a saline-ether procedure. The PAF-fixed specimen, however, cannot be used for permanent stain preparations, and a PVA-fixed specimen should also be collected. Trials in the NCDC laboratory with the PAF-fixative have been satisfactory.

Cultures—Gleason *et al.* (1960) have developed an egg-extract culture medium for shipping *E. histolytica* trophozoites in fecal specimens or sigmoidoscopic material from the field to the laboratory. Screw-cap tubes are used to facilitate mailing. In this medium at room temperature, trophozoites may remain viable up to 4 days, enabling the laboratory to obtain cultures of living trophozoites. For best results, freshly passed feces should be inoculated into the medium. Details of preparation and use are given in the section on Techniques.

Helminths—Adult worms or proglottids of tapeworms are passed with or without fecal material. They can be picked out of feces and placed in a bottle of saline or water or in 70 percent alcohol or 10 percent formalin. If they are to be sent directly to the laboratory, they should not be preserved, since preservation without prior relaxation may cause the organism to contract or may distort it.

Mailing Specimens—Parasitologic specimens sent through the mails are usually preserved and thus are nonviable and noninfectious. Nevertheless, postal regulations require that laboratory and clinical specimens be packed in special containers and in such a manner that material does not leak during transit. Extracts from the United States Postal Manual governing shipment of harmful matter will be found on pages 15-17. The shipping of parasitologic specimens should conform to these regulations. Full responsibility for violations of the law, rests with the mailers.

Double mailing containers consisting of a heavy cardboard outer cylinder and a metal inner cylinder are recommended for shipping parasitologic specimens (Figure 2). Several sizes are available. The outer cylinder, routinely used by NCDC, is 6⅝ inches by 2¾ inches; the inner cylinder is 6⅛ inches by 2½ inches. The vials of preserved feces or the culture tubes should be sealed with adhesive tape around the top to prevent leakage, wrapped in suitable absorbent packing material (cotton or ordinary toilet tissue are satisfactory), and placed in the inner cylinder. Identifying information can be wrapped around the inner cylinder which is inserted in the outer cylinder.

Prepared slides, such as stained fecal smears or mounts of helminths, do not have to meet these postal requirements and can be shipped in cardboard holders, slide boxes, or other suitable containers if they are wrapped carefully to prevent damage or breakage. At NCDC, however, the double cylinders used for vials are also preferred for shipping slides, since they offer better protection than most other types of mailing containers. Slides can be adequately wrapped in facial tissue, toilet tissue, or other soft paper. Toilet tissue, however, makes an excellent wrapping if the following method is used. Place a slide on the tissue and fold the paper over once or twice to surround the slide. Place a second slide on top of the wrapped slide and roll the whole packet over again (Figure 3). In this way, the slides are protected during transit and can be easily unrolled from the packing by the receiver.

Figure 2.
Vials and Container for Shipping Fecal Specimens

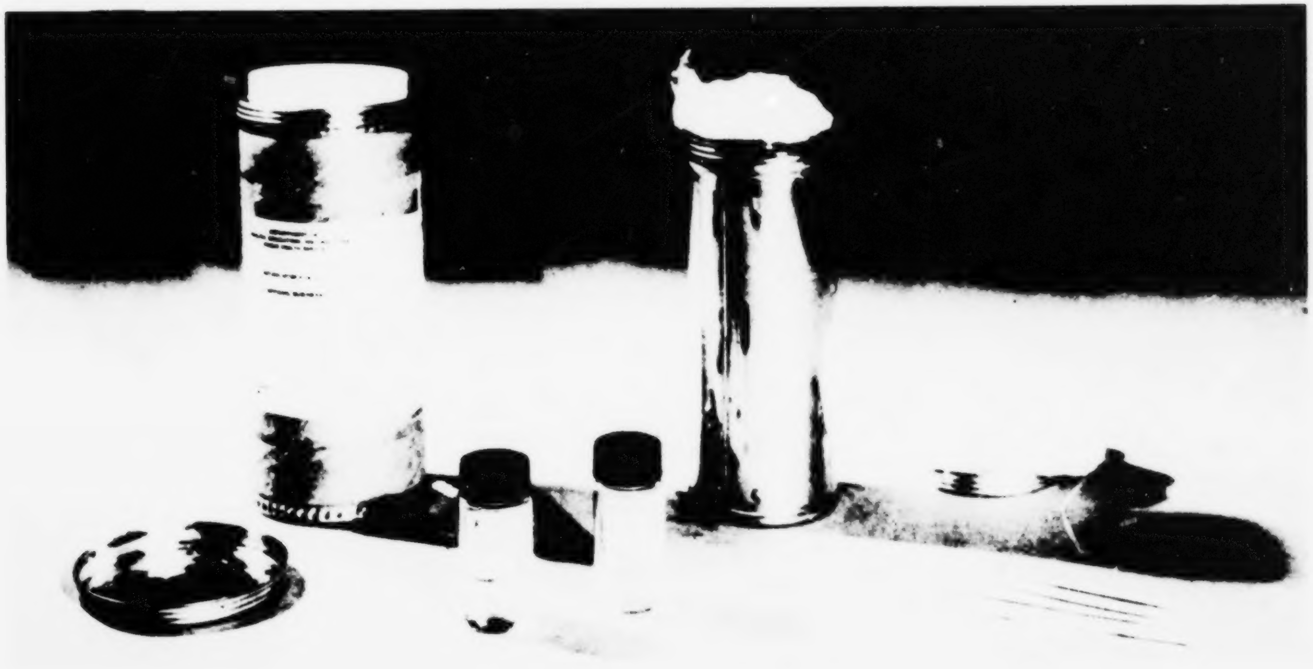
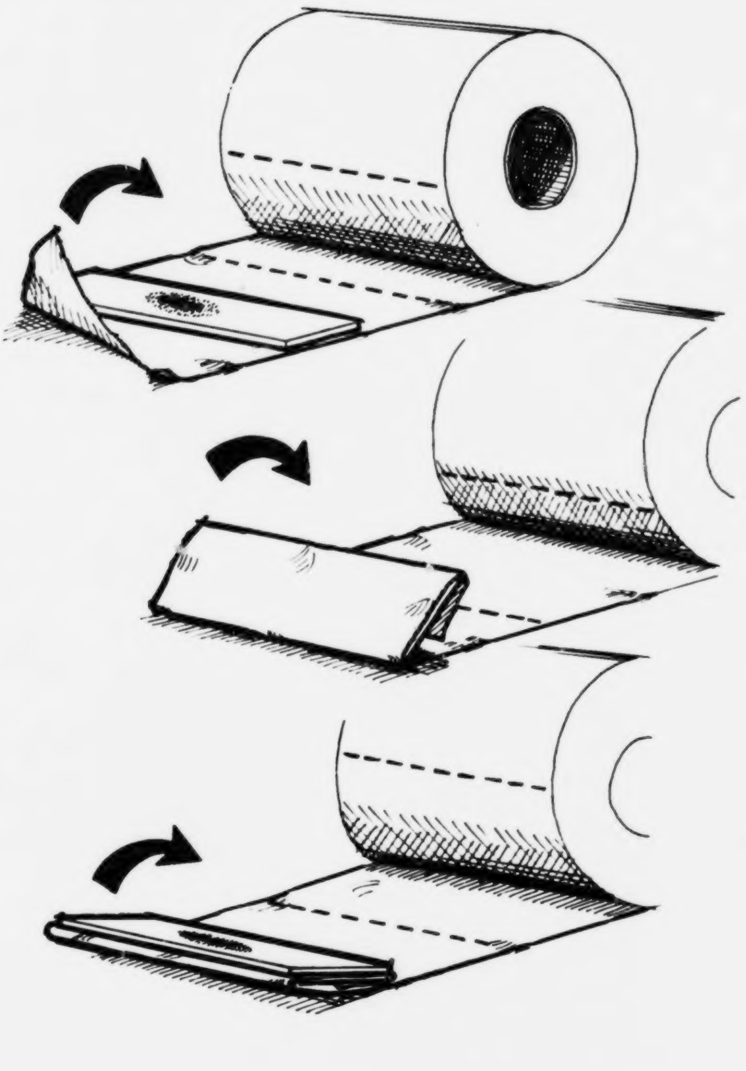


Figure 3.
Method for Wrapping Slides for Shipping



Broken or damaged specimens are of little or no value to the recipient laboratory. Therefore, persons shipping specimens to central laboratories, or elsewhere, should be especially careful to pack the specimens with adequate "cushioning" or protection. Furthermore, the outer container should be clearly addressed to insure its arrival in the proper laboratory. A second label should be placed on the inside cylinder in case the outer cylinder is damaged during transit. Before packing, the sender should make certain that each specimen is properly and completely labelled and tightly capped or sealed and that all essential information accompanies it.

SPECIMENS OTHER THAN FECES

Certain of the intestinal parasites pass diagnostic stages in material other than feces and occasionally intestinal parasites, such as *E. histolytica*, may be located in the liver, lungs, intestinal mucosa, or other tissue. These specimens are usually examined in local laboratories, but if they are to be mailed, they must be packed according to postal regulations as described for fecal specimens.

SPUTUM—Sputum specimens should be collected in suspected cases of paragonimiasis. Although *Paragonimus westermani* eggs are commonly found in sputum, in many cases they may be present in the stools (Komiya and Yokogawa, 1953), and for this reason, fecal specimens as well as sputum specimens, should be examined. Pulmonary amebiasis and echinococcosis possibly may also be detected by examining sputum specimens. In cases of suspected amebiasis, sputum should immediately be examined for trophozoites or preserved in PVA-fixative for staining later. Trophozoites of *E. histolytica* must be differentiated from those of *Entamoeba gingivalis*, a commensal parasite of the mouth that might be present. If examination must be delayed, sputum for diagnosis of paragonimiasis or echinococcosis may be preserved in 10 percent formalin.

URINE—Urine specimens are used in the diagnosis of *Trichomonas vaginalis* and *Schistosoma haematobium* infections.

The optimal urine specimen for revealing eggs of *S. haematobium* has been shown to be the one passed about or shortly after noon rather than the previously recommended early morning specimen (Stimmel and Scott, 1956). Furthermore, eggs are more frequently present in the last few drops of the specimen than in the first portion voided and are more likely to be found in urine containing blood or pus (Belding, 1965). Since the passage of schistosome eggs fluctuates, repeated daily examinations must often be performed to demonstrate the eggs.

In examinations for *T. vaginalis*, fresh urine specimens, preferably the first portion of voided urine (Roth, 1944), should be used. Old specimens are not satisfactory, since the flagellates may be dead or immotile or the urine may become contaminated with a species of *Bodo*, a non-parasitic flagellate, which might be confused with *T. vaginalis*.

ANAL SWABS—Anal swabs are the usual means of detecting *Enterobius vermicularis* infections, since the female worm ordinarily deposits eggs on the perianal region rather than within the intestinal tract. Two types of preparations are commonly in use: the cellulose tape slide preparation (Figure 13, Technique Section) (Graham, 1941; Brooke *et al.*, 1949) and the vaseline-paraffin swab (Markey, 1950). Because of the peculiar migratory habits of the female, these swabs are best collected either between the hours of 9 p.m. and midnight or early in the morning before defecation or bathing. Since migration of the females may not occur every day, examination of a single swab will miss a significant percentage of the pinworm infections; and, therefore, repeated collections should be made. Sadun and Melvin (1956) demonstrated that a single cellulose-tape examination revealed only 55 percent of the infections and that 6 consecutive daily collections were necessary to pick up essentially all of the infections. For practical purposes, 3 consecutive daily collections might be routinely examined, since these authors found that this number revealed 90 percent of the cases. Either type of swab may be stored in the refrigerator, without appreciable damage, for as long as 2 months before it is examined.

Anal swabs are also used for collecting *Taenia* eggs, especially in *T. saginata* infections where proglottids may migrate out of the anus (Rijpstra *et al.*, 1961). They can be collected with a cellulose-tape swab like that used for *E. vermicularis* eggs.

VAGINAL and URETHRAL SWABS—Vaginal swabs or scrapings and urethral swabs may be collected for diagnosis of *T. vaginalis* infections. In female patients, a sterile, non-lubricated dry speculum should be used, and the patient should not douche for 3 to 4 days before the specimen is collected (Perl *et al.*, 1956). If the material cannot be examined in the collecting room, it should be placed in a tube containing a small amount of saline and sent directly to the laboratory for examination. In addition, portions of the exudate may be inoculated into culture media, preferably trypticase-liver-serum medium, and smears for staining may be prepared and placed immediately in Schaudinn's fixative. If the examination will be delayed or if the specimen must be transported to a central laboratory, some of the material should be put into PVA-fixative for future staining.

DUODENAL DRAINAGE—In infections with *Strongyloides stercoralis* and *Giardia lamblia*, duodenal drainage often reveals organisms when the stool specimens are negative (Jones and Abadie, 1954; Gondard, 1956); therefore, when diagnosis cannot be established by fecal examinations, duodenal drainage material should be collected.

SIGMOIDOSCOPIC MATERIAL—When, in suspected cases of amebiasis, stool specimens are negative, material may be obtained from the intestine by sigmoidoscopy. If a patient has had a recent bowel movement when he reports to the clinic, sigmoidoscopy may be performed immediately. If a spontaneous bowel movement does not occur, a cathartic may be given. In this case, sigmoidoscopy must be delayed 2 or 3 hours after purgation. A cleansing enema should not be given, since it may remove organisms from the area which can be viewed through the sigmoidoscope.

A serologic pipette (1 ml) with a rubber bulb should be used to aspirate material from visible lesions and the mucosa. This method is preferable to obtaining material with a cotton swab. The pipette is quite satisfactory for moist lesions; but it is less satisfactory for drier ulcers that are not covered by readily removable exudate (Wilmot, 1962). For these, and moist lesions as well, a curette or Volkman spoon may be used to gently scrape pathologic areas or the mucosal wall (Manson-Bahr, 1944). Bronchial biopsy forceps have also been used successfully (Wilmot, 1962).

ASPIRATES—Liver abscesses suspected of being caused by *E. histolytica* are sometimes drained, and the aspirate is examined for trophozoites. Organisms are located chiefly in the peripheral area of the abscess and aspirates from the center portion are less likely to reveal trophozoites. The Amoebiasis Research Unit, Durban, South Africa (Freedman *et al.*, 1958; Elsdon-Dew, 1960) recommended the following procedure. At least 2 portions of exudate should be removed. The first portion, usually yellowish-white, seldom contains amebae. Later portions, reddish in color, are more likely to contain organisms, and the portion containing material from near the wall is most likely to be positive.

BIOPSIES—Biopsied material is obtained for diagnosis of parasitic infections such as trichinosis, schistosomiasis, amebiasis, and, occasionally, larva migrans. Trichinosis infections are more commonly diagnosed by serologic methods, but in some cases tissue may be examined directly for the presence of larvae. Small portions of skeletal muscle, usually the deltoid or gastrocnemius, may be removed and examined. Tissue from the rectum, colon, and bladder can be obtained for diagnosis of schistosomiasis (Ottolina and Atencio, 1943; Erfan and Talaat, 1947; Gelfand, 1948; Lichtenberg and Valladares, 1955), and punch biopsies of rectal tissue (Juniper *et al.*, 1958; Manson-Bahr and Muggleton, 1957) or liver biopsy tissue (Nelson *et al.*, 1955; Doxiades *et al.*, 1961; Wilmot, 1962) may be used in the diagnosis of amebiasis. In suspected cases of larva migrans, liver biopsy may be resorted to for diagnoses, although the chances of detecting larvae in small liver biopsies are somewhat remote.

Biopsy is not recommended for echinococcosis because of the danger of complications resulting from spilling or leaking hydatid fluid into the surrounding tissue.

Part 124

NONMAILABLE MATTER

124.1 INTRODUCTION

.11 DESCRIPTION. Nonmailable matter includes all matter which is by law, regulation, or treaty stipulation prohibited from being sent in the mail or which cannot be forwarded to its destination because of illegible, incorrect, or insufficient address.

.12 APPLICABILITY. The harmful or objectionable things identified or described in this part are some of the matter which may not be sent through the mail, as a matter of absolute prohibition. See part 125 for matter mailable only under special rules or conditions. Notwithstanding any statement contained in part 124, which covers only some of the items prohibited in the mail, the burden rests with the mailer to assure that he has complied with the law. In addition to the nonmailable items mentioned in this part, certain other articles are prohibited in the mail to military post offices overseas (part 127).

.13 PENALTIES FOR VIOLATION. Severe penalties, by fine or imprisonment, or both, are provided for persons who knowingly mail or cause to be mailed, any matter which has been declared nonmailable under law.

.14 NONCONFORMITY WITH POSTAL REGULATIONS. Regardless of its nature, matter may not be mailed in any form if done in violation of postal regulations for such reasons as failure to pay postage, improper size or weight, improper permits, improper addresses, etc.

.15 RESPONSIBILITY OF MAILER. When mailers are in doubt as to whether any matter is properly mailable, they should ask the postmaster. Even though the Post Office Department has not expressly declared any matter to be nonmailable, the mailer of such matter may be held fully liable for violation of law if he does actually send nonmailable matter through the mail.

124.2 HARMFUL MATTER

.21 GENERAL PROVISIONS OF LAW

Any articles, compositions, or materials, which may kill or injure another, or injure the mail or other property, are nonmailable. This includes but is not limited to:

a. All kinds of poison or matter containing poison.

b. All poisonous animals, except scorpions (see 125.35), all poisonous insects, all poisonous reptiles, and all kinds of snakes.

c. All disease germs or scabs.

d. All explosives, flammable material, infernal machines, and mechanical, chemical, or other devices or compositions which may ignite or explode.

Part 125

MATTER MAILABLE UNDER SPECIAL RULES

125.1 LEGAL RESTRICTIONS

.11 HARMFUL MATTER

.111 Certain items barred from the mail, as set forth in part 124, may be mailed if prepared and packaged in accordance with this part. These are items not outwardly or of their own force dangerous or injurious to life, health, or property.

.112 This part covers generally some of the more common situations; however, the burden rests with the mailer to assure that he has complied with the law and that anything shipped by him has been properly prepared and packaged. The ordinary test of adequate preparation and packaging is whether the contents of a parcel are safely preserved under ordinary hazards of mail handling and transportation.

.113 Products, materials, and devices are created or modified with such frequency that the Post Office Department is unable to issue general rulings in advance to govern adequate preparation and packaging. Any mailer may, however, request the Post Office Department, in advance, for a specific ruling as to mailability of his item. The request should be addressed to the local postmaster, who will forward it to the Classification and Special Services Division, Bureau of Operations, Washington, D.C. 20260.

125.1 LEGAL RESTRICTIONS

.122 Any special conditions or limitations placed on transportation or movement of certain things shall govern admissibility to the U.S. mail, when imposed under law by the U. S. Department of the Treasury; U. S. Department of Agriculture; U. S. Department of Commerce; U. S. Department of Health, Education, and Welfare; Interstate Commerce Commission; or any other Federal department or agency having legal jurisdiction.

.13 PENALTIES

Severe penalties of fine or imprisonment, or both, are provided by law, for anyone who knowingly deposits for mailing or delivery, or causes to be mailed or delivered, anything declared nonmailable under law. Failure to comply with the regulations of the Postmaster General, as prescribed in this part, as to matter otherwise nonmailable, constitutes a violation of law.

125.2 CONDITIONS FOR MAILING

.21 GENERAL NATURE OF PRECAUTIONS REQUIRED

.211 The restrictions against mailing of harmful matter, from which relief is granted by this part, are intended to prevent damage or harm to postal and transportation personnel, to prevent damage or destruction of other mail and of property, to avoid obnoxious odors, and to prevent the spread of disease and infection. Special preparation and packaging are required to protect against such contingencies.

.212 Basic precautions, covered generally in this section, relate to the inner containers holding the harmful matter, internal cushioning and protection, and exterior packaging and marking.

.22 LIQUIDS (NONFLAMMABLE) AND POWDERS

.221 Precautions shall be taken in the case of liquids, pastes, salves, ink powders, pepper, snuff, or other pulverized materials against damage to mail and property from leakage and against caustic, irritant, toxic, or soiling effect on mail handling personnel.

.222 Containers shall meet any applicable Interstate Commerce Commission or other Federal specifications. Closures must effectively seal the contents against leakage. Friction tops must be fastened with solder, clips, or otherwise so that they will not come off under impact.

.223 Glass or other breakable containers of liquid must be packaged to withstand handling enroute. The container shall be cushioned inside the carton to absorb shock and impact. Where feasible, absorbent material shall be used, to take up all the liquid in case of breakage.

125.7 IDENTIFICATION AND MARKING

.71 **IDENTIFICATION OF CONTENTS.** The identity or nature of contents of anything mailed under any of the provisions of part 125 shall be stated plainly on the outside of the parcel, as a condition of mailing.

.72 **IDENTIFICATION OF MAILER AND ADDRESSEE.** The full name and address of both the mailer and the addressee shall be written in ink, rubber-stamped, or pasted on the outside of any package whose mailing is covered by part 125.

.73 **LABELS.** Any labels required under Federal law or under any regulations issued by any Federal agencies pursuant to Federal law shall be pasted to the outside of the parcel.

Subchapter 220 *POSTAL UNION MAIL*

Part 221

CONDITIONS APPLICABLE TO ALL CLASSES

221.1 PREPARING AND ADDRESSING

.11 **PREPARING**

.111 Senders must prepare articles securely, especially if they are for distant countries. International mail is handled more often and subjected to greater pressure and friction than domestic mail, hence it must be enclosed in strong envelopes or other wrappings.

221.3 PROHIBITIONS AND RESTRICTIONS

.325 ***Perishable Biological Materials.*** Perishable biological materials, including those of pathogenic nature, when sent in the postal union mail are accepted only as LETTER PACKAGES. The following conditions apply:

c. *Packaging*

(1) Perishable biological material not of a pathogenic nature must be packed in a nonporous container surrounded by sufficient absorbent material to take up all the liquid and must be placed in an outer protective container where it should fit tightly to avoid any shifting.

(2) Perishable biological material of a pathogenic nature must be packed in a tightly closed bottle or tube or heavy glass wrapped in thick absorbent material rolled several times around the bottle or tube and tied at the ends, sufficient in quantity to absorb all the liquid;

METHODS OF EXAMINATION

MACROSCOPIC

When unpreserved stool specimens are received in the laboratory, they should be examined macroscopically to determine the consistency of the specimen and the presence or absence of proglottids, whole worms, blood, mucus, and other abnormal conditions. Four terms are generally sufficient to designate the consistency of the specimens: watery (W), loose (L), soft, (S), and formed (F). Since (if specimens are of the same age), the more fluid specimens have a greater likelihood of containing protozoan trophozoites, they should be examined in the order listed above. Figure 4 demonstrates the categories of consistency and the distribution of cysts and trophozoites. Distribution of eggs and larvae is less affected by the consistency of the feces. The age of the specimen should also be noted. Recording the consistency and age of the stool specimen in the laboratory record and in the report will permit the laboratory director and the physician to better evaluate results of the examination. Since trophozoites are more or less fragile structures, they die fairly soon after passage from the body, but cysts, which are more resistant, survive for longer periods. Thus, a report of "no parasites found" on a "watery" specimen 24 hours old may be meaningless with regard to the intestinal protozoa; on the other hand, a similar report on a "formed" specimen 24 hours old would have greater significance.

In some cases, macroscopic examination for helminths may reveal organisms which might be overlooked if only a microscopic examination were made. Small helminths, proglottids, or scoleces may be demonstrated by comminuting a small amount of specimen with water and slowly straining the suspension in small portions through a piece of screen wire, a sieve, or a layer of gauze. A small-mesh sieve (30-50 mesh) is useful for this purpose. The sediment remaining on the screen from each portion is washed by running a slow current of water over it. A large hand lens is useful in examining the cleansed debris. Collecting the strained sediment in a shallow pan, the bottom of which has been painted black, or in a glass dish held

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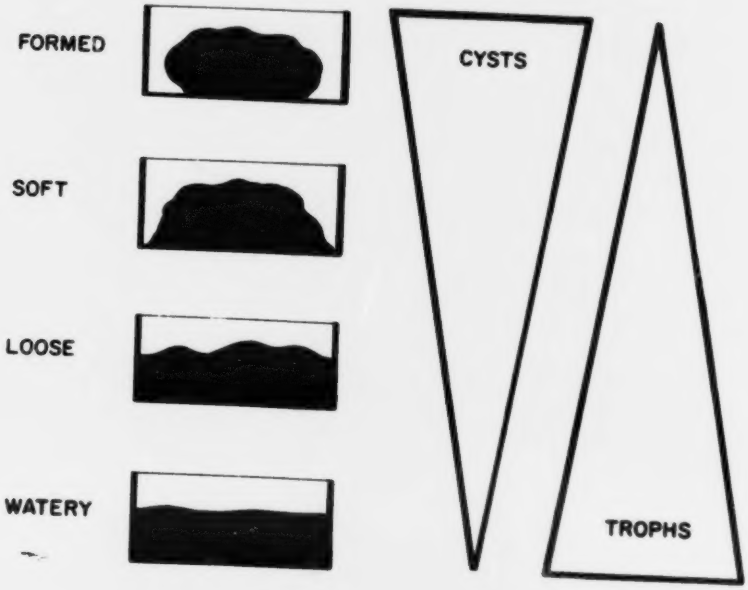
over a black surface will facilitate the examination by increasing the contrast of organisms against the background. Small worms and segments may be picked up with an applicator stick or a camel's hair brush, placed in a drop of saline on a slide, and examined.

Figure 4.
Consistency of Feces and Distribution of Cysts and Trophozoites



MICROSCOPIC

The microscopic examination of feces may reveal helminth eggs, larvae, and small adults; protozoan trophozoites and cysts; discharged body cells, such as macrophages, indicative of bacterial infection, and epithelial cells; red blood cells sometimes indicative of ulceration, or of hemorrhagic or vascular lesions; various inanimate objects, such as Charcot-Leyden crystals which occasionally are correlated with amebiasis and other parasitic infections; fungi such as *Blastocystis hominis;* plant cells, including stone cells, parenchymal cells, starch granules, and pollens, which may be confused with cysts or eggs; and plant fibers and plant or animal hairs which may be confused with nematode larvae.

MICROSCOPY—Since the differentiation of intestinal parasites depends on observing the morphologic characteristics of the diagnostic stages, having a good binocular microscope and a properly aligned lamp is essential. The make of microscope is not an important factor. The instrument should be equipped with 3 objectives: low power—10X (16 mm); high, dry—44X (4 mm); and oil immersion—97X or 98X (1.8 mm). A 50X or 60X oil immersion lens is desirable for scanning stained slides. Both 5X (or 6X) and 10X oculars should be available. In many cases the use of a lower power (5X or 6X) facilitates examination. Special oculars, such as 12.5X wide-field and 10X wide-field, may also be used.

Most of the microscopes equipped with built-in illuminators are satisfactory for parasitology work, especially if a voltage regulator is included. For microscopes requiring a separate illuminator, the best type of lamp is one with a condenser system, regulatory iris diaphragm, separate removable filters (preferably ground, white glass and blue glass), and the maneuverability to be raised or lowered and focussed at different angles. A bright light (100-watt bulb) is usually used in this type of lamp. Certain other kinds of lamps that take a 75- or 100-watt bulb and either have or can be fitted with filters are also satisfactory. However, the small box-type lamp often used as a substage illuminator may not give enough light to adequately observe details in fecal specimens. The need for blue, clear glass and white, ground glass filters must be emphasized, since the bright yellow light obtained without these can cause difficulties in seeing the minute details necessary for species identification. The microscope and light source should be aligned for optimum illumination. In addition, the substage condenser must be positioned properly. Suggestions for adjusting the microscope in examining parasitologic materials are given in the Techniques Section and in Figure 7.

The observation of structural details, particularly in wet mount fecal suspensions, is facilitated by reducing the light or regulating it until elements in the specimen are clearly visible. A too-bright light will obscure many structures. Begin with a lower intensity, increasing it until satisfactory illumination is obtained. Best results are achieved by leaving the substage condenser in the optimal position and regulating the light intensity by means of the iris diaphragm. Improper focussing of the condenser and improper illumination may cause difficulties in examining specimens. Attention to these two factors will increase the observer's efficiency.

Furthermore, the microscope should be kept clean and free from dust, and the surface lenses of both objectives and oculars should be cleaned frequently with a good grade of lens paper. Dust particles may interfere with the observation of the structural details of organisms. Oil should be removed form the oil immersion objective as soon as the examination is finished. Oil left on the lens may form a film that is difficult to remove or seep into the objective causing a cloudy or hazy appearance. A good quality, low viscosity immersion oil, such as Cargille's grade A, low viscosity, is recommended. Cedar, mineral, or similar oils may damage both the slide preparations and the microscope. Cedar oil tends to produce a hardened film over the lens, and, despite cleaning efforts, mineral oil frequently seeps into the objective. Excessive use of xylol to clean oculars or objectives should also be avoided.

Size is an important criterion in identifying many parasite species. A calibrated ocular micrometer disc, placed inside the ocular of the microscope, should be used for accurate measurement of organisms. The technique for calibrating the micrometer is given in the section on Techniques and in Figure 8.

PROCEDURES FOR FECES—The procedures used for microscopic examination vary, depending on many factors such as the age of the specimen, the organism suspected, the type of equipment available, and the time that can be spent. No one technique is 100 percent effective in revealing organisms. The technologist should be familiar with several methods and their advantages and disadvantages. Microscopic techniques include direct wet mount examinations, concentration, permanent staining, and cultivation procedures, and special methods for certain infections.

Wet Mounts—Wet mounts made directly from stool specimens require the least equipment of any of the available examination methods and are the simplest to prepare. By using various solutions, all types and stages of organisms encountered can often be identified. For wet mounts of fresh or unpreserved feces, saline and iodine mounts are routinely prepared. If preserved feces is used, the formalin preservative serves as a diluent and additional saline is usually not necessary for the unstained mounts. In some cases, special temporary stains may be used to demonstrate parasite structures.

For routine examination, a systematic scanning of the entire preparation with optimal illumination is recommended. This method reduces the chances of missing scarce organisms that might be present and thereby increases the value of the direct wet mount in diagnosis.

Saline Mounts—Physiological saline constitutes the most normal environment for all types and stages of organisms which may be encountered in the stool specimens. Eggs and larvae can be readily detected and identified in saline mounts. Both the trophozoites and cysts of intestinal protozoa appear highly refractile and the trophozoites (if living) may exhibit characteristic locomotion. Because of these properties, organisms can be more easily spotted in saline than in any other type of preparation, even with the low power objective. Some structures, such as the chromatoid bodies in cysts and red blood cells in trophozoites, are more easily seen in saline than in stained temporary mounts, although others, such as nuclei, may be invisible.

Temporary Stains—Although helminth eggs and larvae can be identified more easily in unstained mounts, the use of various temporary stains will aid in identifying protozoa.

Iodine solutions are used primarily for staining cysts to determine the number and structure of the nuclei. The solutions will also stain glycogen masses, and thus they help identify *Iodamoeba bütschlii* cysts. The iodine solution must be of the right concentration, preferably about 1 percent strength, to insure good results. Dobell's or D'Antoni's iodine solutions are recommended. Lugol's iodine can be used but must be diluted 1:5 for fecal preparations. Undiluted, it is too strong and will cause the feces to clump perhaps obscuring or entangling organisms. A too-weak iodine solution, such as Gram's, will not stain the organisms properly.

The nuclei of amebae trophozoites, like those of cysts, are indistinct or invisible in saline preparations. However, iodine is unsatisfactory for the trophozoite stage, since it may distort the entire organism. Therefore, various staining solutions have been advocated to assist in identifying amebic trophozoites.

Quensel's stain (Svensson, 1935), which Quensel first described in 1918 for staining bacteria, stains the nuclei and helps to differentiate 3 genera of amebae—*Entamoeba*, *Iodamoeba*, and *Endolimax*. Unfortunately, it will not stain *Dientamoeba fragilis* and may not be adequate for differentiating *E. histolytica* and *E. coli.* Since it will not stain living or unpreserved cysts, it helps the technician to distinguish rounded-up trophozoites from cysts.

Merthiolate-iodine-formaldehyde (MIF) solution stains well both the cysts and trophozoites of all the amebae except *D. fragilis* (Sapero and Lawless, 1953).

Nair (1953) showed that the visibility of the nucleus of the *E. histolytica* trophozoite depends on the pH of the suspending solutions and has suggested acetate or phthalate buffers in the lower pH ranges for bringing out nuclear details. Thus, one need not have the more complicated staining solutions. Methylene blue, pyronin, and other available stains in a buffered solution of the desired pH will serve the purpose.

Concentration—When organisms are rare or not found in direct mounts, the feces may be concentrated. Many reliable techniques have been developed and this type of examination has become a part of the laboratory parasitologic routine because it increases the chances of detecting organisms and insures a more thorough examination. The concentration procedures are applicable to helminth eggs and larvae and protozoan cysts, but not to protozoan trophozoites. The latter are usually distorted or destroyed during the process.

Concentration techniques are generally divided into flotation procedures and sedimentation procedures. The specific techniques used will depend in part on the organism to be searched for and the type of material to be examined. To be successful, the solutions used in these methods must have certain characteristics: for flotation techniques, the solution must float the majority and preferably all of the eggs, larvae, and cysts in the specimen; for sedimentation methods, the solution must permit the settling of all of these elements.

Flotation—Bass (1910) introduced this type of concentration for the recovery of hookworm eggs. The procedures make use of a solution which has a higher specific gravity than the organisms to be floated so that the organisms rise to the top and the debris sinks to the bottom. Eggs and cysts usually have a specific gravity ranging between 1.05 and 1.15, although a few eggs, such as *Clonorchis,* *Opisthorchis*, and related species, have specific gravities over 1.20 and cannot be concentrated by the usual flotation techniques.

The most common techniques used are the zinc sulfate centrifugal flotation (Faust *et al.*, 1938) and the brine flotation (Willis, 1921). The zinc sulfate solution is approximately a 33 percent aqueous solution of specific gravity 1.18 or 1.20. The former density is commonly used with fresh specimens and the latter with formalinized specimens. The density should be checked for accuracy with a hydrometer, since, because of impurities, a 33 percent solution may not give the required specific gravity. The sodium chloride or brine is a saturated aqueous solution prepared from crude salt and has a specific gravity of 1.20. These solutions may be stored in tightly stoppered bottles, but, if used over long periods, the specific gravity of the zinc sulfate should be rechecked from time to time.

The zinc sulfate technique is an effective procedure revealing both protozoan cysts and helminth eggs and larvae in unpreserved specimens (Shrivastav, 1954; Guinn, 1959). (See section on Techniques.) With various slight modifications, it has been used successfully in numerous laboratories. However, operculated eggs and those of the schistosomes are not recovered. Furthermore, in some instances distortion of protozoan cysts may occur. Harper (1964) has modified this method by preparing an initial formalin feces suspension. The tube of material is centrifuged, the supernatant fluid decanted, and the sediment used for the usual concentration technique. Preparation of a formalinized fecal suspension permits delayed examinations of the specimen without degeneration or deterioration of the organisms. The formalin also reduces distortion in the cysts.

The brine flotation is particularly recommended for recovering hookworm eggs, but it is also useful in recovering other helminth eggs especially those of *Ascaris*, *Trichuris*, *Taenia*, and *Hymenolepis nana*. (See section on Techniques.) Operculated eggs, schistosome eggs, and protozoan cysts will not be recovered with this method. Therefore, because of its restricted use, this technique should not be used as a primary routine diagnostic procedure.

Sedimentation—In these techniques, the organisms are sedimented either by gravity or centrifugation. Feces may be suspended in tap water alone, or various solutions can be used to clarify the suspension, free the organisms from the debris, and aid the settling of these elements. The sedimentation procedures are useful in concentrating protozoan cysts and helminth eggs and larvae, including the schistosome and operculated eggs. By substituting saline for the tap water, protozoan trophozoites may occasionally be recovered.

The simple gravity sedimentation is time consuming and somewhat less effective than other methods but does possess certain advantages. (See section on Techniques.) By using saline, viable eggs of the schistosomes may be freed from the fecal debris and recovered in the sediment. If examinations of the sediment fail to reveal suspected schistosome infections, the material can be suspended in chlorine-free water in a narrow mouth flask, such as an Erlenmeyer flask, and allowed to stand overnight. Viable eggs will hatch and the miracidia will be found swimming in the upper layers of the solution. (See section on Techniques.) The gravity sedimentation method is probably one of the most practical for obtaining immature, mature, and degenerate eggs of the schistosomes (Maldonado and Acosta-Matienzo, 1953). The time involved may be shortened by centrifuging the fecal suspension.

Various sedimentation techniques use chemical solutions, such as hydrochloric acid, acetic acid, or sodium sulfate, to clarify the material and liberate the organisms from the fecal debris. Telemann (1908) devised the first of these techniques, an acid-ether method which is particularly useful in recovering schistosome eggs. Many modifications of this method have been developed, but none are satisfactory for recovering protozoa. Alicna and Fadell (1959) reported that acid-ether techniques were more effective than either zinc sulfate or formalin-ether methods in recovering helminth eggs and larvae.

The substitution of formalin for the acid or sodium sulfate made possible an effective sedimentation technique for revealing both protozoa and helminths (Ritchie, 1948). This formalin-ether (FE) sedimentation technique is satisfactory for both unpreserved and formalin preserved specimens and has the added advantage of recovering operculated eggs and those of schistosomes as well as other helminths. A modification of this method (Maldonado *et al.*, 1954) that uses Triton NE (a non-ionic detergent) in the formalin increased the effectiveness of the FE technique in recovering helminth eggs. Ritchie *et al.* (1960) found that the pH of the formalin affected recovery of cysts ~~and eggs.~~ *Ascaris* and *Schistosoma japonicum* eggs were recovered best at pH 10.0, but hookworm egg recovery was best at pH 4.0 and pH 7.0. Recovery of *Trichuris* eggs and protozoan cysts was not particularly affected by changes in pH, but pH 7.0 seemed to give the best results. These data suggest that formalin adjusted to a neutral pH might be more satisfactory than unbuffered solutions in recovering organisms.

The FE method, like the zinc sulfate technique, is especially useful as a routine diagnostic procedure. In comparative studies with the zinc sulfate flotation procedure, the FE sedimentation technique has been considered more efficient (Ritchie *et al.*, 1952, 1953). In an unpublished study conducted by NCDC, the FE technique gave slightly better results than the zinc sulfate on both unpreserved and preserved specimens. In general the cysts are more normal in appearance and are more easily identified in the preparations from FE concentrations. Since formalin fixes the organisms, the nuclei and chromatoid bodies of cysts are more distinct than in the zinc sulfate method. Furthermore, less distortion of small cysts, such as *Endolimax nana* or *Entamoeba hartmanni*, occurs. Occasionally, with zinc sulfate flotations, cysts, especially those of *E. nana*, will appear "caved-in" on one side. Moreover, if the flotation stands for some time, many organisms will sink to the bottom of the tube and examining the surface film may cause infections to be overlooked. The FE sedimentation not only recovers a wider range of organisms but also permits keeping the tubes longer without affecting the results of the examination. After pouring off the supernatant, the tube can be stoppered to prevent drying and the sediment can be examined later. If the quantity of fluid with the sediment is minute, a drop or two of saline or formalin should be added. Tubes left for several hours or overnight should be placed in the refrigerator to prevent drying. The FE technique, however, is not as efficient as the zinc sulfate method for detecting *G. lamblia* cysts, *H. nana* eggs, or *I. bütschlii* cysts.

The MIF ether concentration (MIFC) technique proposed by Blagg and co-workers (1955) is essentially the FE technique with MIF solution substituted for formalin. However, the alcohol and acetone in the tincture of merthiolate apparently reduce the specific gravity, and this reduction may increase the concentration of eggs. The MIFC technique can be performed on either fresh specimens or those preserved in the MIF solution.

Regardless of which technique is used, the mounts should be carefully and systematically examined.

Technical Errors—The efficiency of any concentration procedure is dependent to a great extent on the capability of the technician performing the technique. Numerous technical errors which would make the results invalid can occur. For example, the sample of specimen used may be too small for adequate examination or too large to be properly concentrated. The suspensions may not be thoroughly comminuted or the supernatant may be carelessly decanted resulting in loss of material. In the flotation methods, the specific gravity of the solutions may be too low to float the organisms resulting in poor recovery, or too high, resulting in distortion of cysts and other stages. Carelessness in handling flotation specimens may disrupt the surface film and force some of the organisms into lower fluid levels.

Permanent Stains—Stained fecal smears are usually prepared for the identification of protozoa. Helminth eggs and larvae may be found on such smears but, often, they are distorted or the stain obscures their differential characteristics. Occasionally, however, special stains or clearing procedures are used in identifying the helminths.

Protozoa—Various authorities have emphasized the importance of using permanently stained fecal preparations for demonstrating and accurately identifying intestinal protozoa (Meyer and Johnstone, 1935; Goldman and Brooke, 1953; Anderson *et al.*, 1953; Brooke *et al.*, 1954; Goldman, 1964; Healy, 1964; and others). In temporary wet mounts, unless indisputably typical characteristics of *E. histolytica* or other protozoa are observed (preferably in the cystic state), identification should be considered only tentative. Confirmation in permanently stained slides is often essential for accurate diagnostic work. Since the procedure need not be time consuming, all specimens found to contain amebae or questionable organisms should be stained. Furthermore, permanently stained fecal preparations may reveal organisms, especially small protozoa, not found by other techniques (Wenrich *et al.*, 1935; Faust *et al.*, 1939; Goldman and Brooke, 1953; Brooke, 1958, 1960; Brooke *et al.*, 1962, 1963a; Melvin and Brooke, 1962; and others). In most instances a stained fecal smear should be examined for a minimum of 15 minutes. If suspicious structures are found, longer examinations may be necessary. By using a low power oil immersion objective (43X or 50X) in place of the usual 95X or similar power, a greater amount of material can be examined. The higher power may be needed, however, for careful study of structural details.

Stained fecal smears afford the most satisfactory method for keeping positive evidence of the intestinal protozoa a laboratory identifies. In view of the existing doubt about the reliability of the available diagnostic characteristics, those responsible for the identification of intestinal protozoa should maintain evidence of their positive findings.

Permanently stained preparations also make it possible to obtain consultation when needed. Even the authorities in the field welcome the opinions of others on certain specimens, and permanently stained preparations enable an individual to obtain assistance more easily. If the clinical laboratory cannot prepare permanently stained slides, specimens preserved in vials of PVA-fixative or dried PVA films can be shipped to a central diagnostic laboratory for processing.

For several decades, many workers have preferred the Heidenhain's iron hematoxylin procedure for staining fecal smears. Although this procedure may take a day or two to complete, it can frequently be incorporated into a busy laboratory regimen more easily than a shorter procedure which requires constant attention. The time needed to perform Heidenhain's procedure can be reduced by heating certain of the solutions. A number of simpler staining techniques which can be completed in less than an hour have been developed: for example, the phosphotungstic acid-hematoxylin stain (Tompkins and Miller, 1947) and the trichrome stain (Wheatley, 1951). In a comparative study, Markell (1956) showed that the trichrome method is satisfactory for diagnostic purposes and many laboratories, including NCDC, currently use it for routine staining.

Kohn (1960) recently developed a staining procedure using chlorazol black in which fixation and staining take place in a single solution. Destaining is not necessary, which simplifies the procedure to some extent. A modification of this technique (Gleason and Healy, 1965) has been found satisfactory for staining protozoa in fecal smears or tissues. Although fresh, unpreserved feces stain well with this stain, PVA-fixed specimens do not. The chlorazol black stain could be of particular value in hospital or clinic laboratories, where fresh specimens are usually obtained, since the steps in the technique are fewer than in other procedures and require less attention.

Specimens fixed in formalin are unsatisfactory for staining because the material will not adhere properly to the slide and the organisms, especially the chromatin portion, stain poorly. Unfortunately, the formalinized feces cannot be mixed with PVA-fixative to overcome the lack of adhesion because the two solutions are not compatible and a cloudy precipitate forms. A technique for staining formalin preserved material has been developed (Jeska and Gentzkow, 1958), but the results are variable and it is of little value in the diagnosis of protozoa.

Arensburger and Markell (1960) described a procedure for concentrating cysts, mixing the concentrates with fresh feces and staining smears by the usual procedures for permanent preparations. (See section on Techniques.) To date no satisfactory procedure for concentrating trophozoites in fecal material for subsequent staining is available.

In preparing permanently stained slides, fecal material may be stained in bulk in test tubes, but the results vary and the degree of staining or differentiation is more difficult to regulate than with smears made directly on the slide. Practice and experience are usually necessary to achieve good preparations.

Helminths—In most instances, helminth adults can be identified macroscopically, or, microscopically in temporary wet mounts without staining. Observation of living, small organisms in saline often provides valuable information. Helminths should always be handled as gently as possible, especially in the living condition, to prevent rupture and distortion. If small enough, they can be sucked up in a wide bore pipette and placed in a dish of saline. Small tapeworms can be handled either with camel's hair brushes, blunted needles, or applicator sticks with edges cut in the shape of thin, flat spatulas. Larger organisms, of course, are not as fragile and if care is taken not to damage them, they can be handled with any suitable blunt instrument. Whole mounts of the worms or proglottids are best examined under a low power magnification. If adult helminths are received frequently, a 3.5X objective on the compound microscope is desirable. A dissecting microscope such as that commonly used in entomology studies or even a good hand lens also may be used.

Special stain procedures are available for helminth adults but are not used routinely to identify the common human intestinal species. In determining nematode morphology, clearing the specimen will usually result in a satisfactory preparation for differentiating structures. The clearing agents commonly used are carbol-xylol (1:3), lactophenol (1:1), beechwood creosote, xylol or glycerin. The specimen should be turned and rolled so that it can be observed at various positions. Cestode proglottids, especially those of the *Taenia* species, may also be cleared for identification. The cleared specimens can be mounted in balsam or synthetic media, such as Permount, for permanent preparations. A PVA-lacto-phenol medium which combines the clearing and mounting processes has been advocated for small nematodes (Rubin, 1951). Modifications of this method have been used successfully in the NCDC laboratories to clear and mount both nematodes and cestode proglottids. (See section on Techniques.)

Hematoxylin or carmine stains are widely used for trematodes or cestodes where species differentiation is a problem. Either Delafield's or Ehrlich's hematoxylin, usually available in most laboratories, will give excellent results. Both are aqueous stains and best results are achieved when very dilute solutions are used. This permits slower penetration, and the specimen can be watched and removed form the solution when the structures become stained. Furthermore, dilute stain solutions acting over a long period (1 to 2 days) will not require a great deal of destaining. Semichon's carmine is also a good stain for routine purposes, especially for tapeworm proglottids.

Directions for clearing and staining procedures will be found in the Techniques section. Additional information is available in various texts such as Guyer, *Animal Micrology;* Pearce, *Histochemistry;* and Lillie, *Histopathologic Technique and Practical Histochemistry.* (See the list of references for publishers.)

Cultivation—Like staining techniques, cultivation procedures are used primarily in the diagnosis of intestinal protozoa, although certain special techniques have been developed for cultivating helminth larvae.

Protozoa—Cultivation has two chief advantages: to increase the number of organisms available for observation and to revive trophozoites that may be degenerating. It is a useful tool for very fresh specimens in which organisms are rare or are not found by other methods (Norman and Brooke, 1955a).

In the diagnosis of protozoan infections, cultivation can be used to advantage with fresh specimens if there are adequate facilities, time, and interest, but it is not a recommended procedure for laboratories which receive specimens by mail (Norman and Brooke, 1955a). The method is applicable to all of the intestinal protozoa except *G. lamblia* and the coccidia species, although *Balantidium coli* usually requires special media. *Giardia* has been successfully grown in a tissue culture medium (Karapetyan, 1960, 1962), but this specialized procedure is not applicable to routine or general laboratory work. Coccidia cannot be satisfactorily cultured.

Several media are available for the cultivation of intestinal protozoa; for example, liver extract (Cleveland and Collier, 1930); modified Boeck and Drbohlav's or LER (Boeck and Drbohlav, 1925; Reardon and Rees, 1939); egg yolk infusion (Balamuth, 1946); and alcohol egg extract (Nelson, 1947). Selecting a single medium and becoming proficient in its use is generally better, however, than using several media. Small quantities of penicillin and streptomycin should be added to most media before inoculation to suppress bacterial multiplication (Spingarn and Edelman, 1952; Norman and Brooke, 1955a). At least 2 tubes of media should be inoculated with different quantities of the specimen. The sediments in the tubes are examined at the end of 24 hours of incubation at 37° C. and, if organisms are not found in the first examination, again after 48 hours. In addition, subcultures into fresh tubes of media may be made at the end of 24 hours. The subcultures should also be examined after 24 and 48 hours' incubation.

Although *E. histolytica* tends to culture more easily than the other species, no satisfactory differential medium that will isolate this pathogen alone from stool specimens is available. For this reason, after the amebae have been successfully established in culture, the necessity of differentiating the species on morphologic characteristics remains. Generally speaking, if the trophozoites demonstrate rapidly progressive locomotion and have an *Entamoeba* type nucleus, they can be safely identified as *E. histolytica*. However, the other species, particularly *E. coli*, occasionally exhibit the same activity. In examining the cultures, stained and unstained wet mounts should be prepared from the sediment in the tubes. The full value of cultivation as a diagnostic procedure will not be realized unless permanently stained preparations are made from the sediments. In a group of 878 fecal cultures, Norman and Brooke (1955b) increased the number of positive identifications from 212 to 391 by examining permanently stained culture sediments preserved in PVA-fixative.

Cultivation may be of assistance in diagnosing trophozoites in the process of degeneration. If the organisms have not deteriorated too much, they may revive in culture and produce progeny with more typical characteristics. Furthermore, if encystation can be accomplished perhaps by incubating for 72 hours, cultivation may be a method for obtaining the more diagnostically reliable cystic stages.

Although, as previously stated, coccidia cannot be cultured, a type of "cultivation" or "maturation" procedure may be used in some cases where immature oöcysts are found in feces and identification of genus is doubtful. Immature oöcysts of *Eimeria* (animal species) and of *Isospora* are often difficult to distinguish, and since *Eimeria* oöcysts are sometimes accidentally ingested and passed in human feces, generic identification may be important. The simplest method of doing this is to allow the immature oöcysts to mature and to determine genus from the mature stage. The procedure is described in the Section on Techniques.

Helminths—Cultivation of helminth larvae is used chiefly in differentiating hookworm and *Strongyloides* larvae. Occasionally, larvae found in feces cannot be specifically identified, or a question of infection with both species may arise. In these cases, cultivation to obtain filariform larvae may be advantageous.

Sand or charcoal cultures are often used, and the larvae are collected by means of a Baermann apparatus (Figure 12) (Baermann, 1917). In this way, larvae free of feces and culture material are obtained. Several modifications of the Baermann apparatus have been made for recovering hookworm larvae (Beaver, 1953; Watson and Al-Hafidh, 1957) and *Strongyloides* larvae (Ferriolle, 1959). This type of apparatus and procedure can also be used for isolating or recovering various nematode larvae in soil samples. However, free-living larvae must be distinguished from parasitic species. Free-living larvae can be killed by a dilute solution of hydrochloric acid; parasitic species are unharmed (Shorb, 1937). (See section on Techniques.) Feces can be cultured on filter paper strips in a test tube, a procedure which is easier and simpler to perform than charcoal or sand cultivation (Sasa *et al.*, 1958). Larvae of *Strongyloides*, hookworm, and *Trichostrongylus* are recovered by this method. It can also be used as a diagnostic procedure when hookworm or *Strongyloides* infections are suspected but eggs or larvae cannot be found by the usual methods.

Directions for culturing larvae and using the Baermann apparatus are included in the Techniques section.

Estimation of Worm Burden

Ascaris, Trichuris, and Hookworm—The physician can often use some indication of the intensity of helminth infections and, for this purpose, quantitative egg counting procedures have been developed for certain of the nematode infections. In infections with *Ascaris*, *Trichuris*, and hookworm, fairly reliable egg counts can be made because the egg production is more or less constant, the approximate daily output per female worm is known, and (as with other helminths) the worms do not multiply in the body. Egg counts provide an *estimate* only of the worm burden and should not be considered an accurate worm count. Nevertheless, counts do furnish the approximate number of worms harbored. The number enables one to classify the infection as light, moderate, or heavy. Fluctuation in numbers of eggs shed, the amount of feces passed, distribution of eggs in feces, and errors in sampling introduce variations in the counts. If performed properly, however, they provide an index of infection which is of value in determining the degree of infection. The counts are used chiefly to estimate the worm burdens in population groups when mass therapy or community control measures are being considered. In individual patients, egg counts may be useful both in indicating the intensity of infection and in following the efficiency of a therapeutic regimen. Whenever possible, therefore, egg counts should be performed on unpreserved fecal specimens in which hookworms, *Ascaris*, or *Trichuris* eggs are found.

Two procedures for making egg counts are currently available: the dilution egg count technique (Stoll, 1923) and the direct egg count (standardized smear) technique (Beaver, 1949a, 1950). Both methods are described in the section on Techniques. Comparison of these two procedures indicates that they are equally satisfactory for routine egg counts (Melvin *et al.*, 1956). The direct smear technique, however, is easier for the average technician to perform than the dilution method and requires less time.

Other Infections—The quantitative procedures are not applicable to other helminths, since either the shedding of eggs is irregular or the eggs are not ordinarily passed in the feces. Counts can be performed, of course, in infections other than those of *Ascaris*, *Trichuris*, or hookworm, but they should be done with the understanding that only very rough estimates of worm budren may be obtained. Sadun (1955) used egg counts as a means of estimating density of *Opisthorchis* infections in various population groups in Thailand;

Stimmel and Scott (1956) made egg counts in an attempt to estimate severity of *S. haematobium* infections; and Bell (1963) devised a method of counting *Schistosoma mansoni* eggs in feces. Sadun *et al.* (1956) have suggested a coefficient of infection as an index to the intensity of *Enterobius* infections in different population groups. The technique can possibly be applied, within limits, to the individual pinworm patient. The method of determining this coefficient of infection is described in the section on Techniques.

OTHER SPECIMENS

Sputum and Urine—The methods of examining feces are also applicable to urine and sputum. Usually simple centrifugation of urine and subsequent examination of the sediment is sufficient to reveal any protozoa or helminths present. Sputum specimens may be satisfactorily concentrated by either acid-ether or formalin-ether techniques or by mixing the material with 3 percent sodium hydroxide and centrifuging.

Anal Swabs—Anal swabs collected for diagnosis of pinworm infections are handled according to the type of preparation used. They should be examined as soon as possible after collection, but if a delay is necessary, they should be stored in the refrigerator. Cellulose tape slides may be examined directly or cleared by adding a drop of toluene or xylol under the tape (Beaver, 1949b), thus, making the debris more or less transparent and the eggs more easily visible. Vaseline-paraffin swabs are handled according to the directions in the Techniques section. Comparison of these two techniques at NCDC indicates that they are about equally efficient. On occasion, when collections are made during the day, the vaseline-paraffin swab reveals a few more infections than the cellulose tape slide.

Cellulose tape swabs are examined for *Taenia* eggs in the same way as for *Enterobius* eggs.

Vaginal and Urethral Material—Vaginal swabs or scrapings or urethral swabs are usually examined for *T. vaginalis* in wet saline mounts immediately after being collected. The presence of the trichomonads is ordinarily sufficient for a species diagnosis and stains are unnecessary. If dried vaginal smears are received, however, they may be stained with Gram's, Wright's or similar stains. The trichomonads are often distorted in the dried preparations and better results are obtained by fixing the wet smears immediately in Schaudinn's fixative or by putting the vaginal material into PVA-fixative and subsequently staining it by the methods used for feces. In the NCDC laboratory, less distortion and better differentiation of the flagellate trophozoites (*Trichomonas* spp. and *Chilomastix mesnili*) have been observed in stains prepared from PVA-fixed specimens rather than from unpreserved specimens.

Vaginal or urethral exudates may be cultured using trypticase-liver-serum medium (Burch *et al.*, 1959). Cultivation is considered a sensitive diagnostic tool for *T. vaginalis* infections, especially in light cases. Furthermore, it may be more convenient than examining direct mounts, since the tubes of media can be inoculated at the time the exudate is collected and examined later. Scrapings or material obtained with a speculum are introduced into the tube of media and incubated at 37° C. Readings are made at 48- and 96-hour intervals. If a sterile cotton swab is used to obtain material, the entire swab is placed in the tube of media.

Duodenal Drainage—Material obtained by duodenal drainage for diagnosis of strongyloidiasis and giardiasis can be examined directly in wet mount preparations. Gondard (1956) has stated that *Giardia* trophozoites are lysed in bile and the specimen should be preserved in formalin unless examined immediately.

Sigmoidoscopic Material—The amount of material obtained is usually very small, especially if the lesions have been scraped rather than aspirated. Direct saline mounts should be made immediately and examined for trophozoites. The material is obtained from the lesion and mixed at once with a drop of saline. Macrophages and epithelial cells will probably be present in the material also and must be differentiated from amebae trophozoites. If organisms or suspected organisms are found, a drop of Quensel's stain or buffered methylene blue solution can be added to stain the internal structures. In addition, a permanent stain can be made and, indeed, may be necessary for positive identification. Since the quantity of specimen is limited, two or three drops of PVA-fixative can be added to the saline mount, mixed thoroughly, and spread into a smear for staining. The smears are dried and stained as for fecal smears. The lesion material often seems to coagulate in the fixative, however, and good smears are difficult to obtain. Juniper (personal communication) has recommended that the material be pressed between two slides, "squashed" as it were, to remove the lumps. In the NCDC laboratory, this procedure has been found satisfactory for obtaining even, uniform preparations. Sigmoidoscopic material can be cultured, but the minute quantity available may cause difficulties in obtaining positive cultures. Generally, if trophozoites are present, they can be detected in wet mounts.

When sigmoidoscopy is performed away from laboratory facilities, the material can be placed in a tube containing a *small* quantity of saline and taken at once to the laboratory. It should be kept warm during transit. It too much saline has been added, the material can be gently centrifuged (about 1,000 r.p.m. for 1 minute) and wet mounts made from the sediment. If the specimen cannot be sent to the laboratory immediately, the material may be put into PVA-fixative for subsequent staining. The recently developed PAF-fixative (Burrows, 1967a), described under the heading "FECES: Storage, Preservation and Shipment," may be useful in preserving sigmoidoscopic material, but it needs to be evaluated for this purpose. Material can also be inoculated into a shipping culture medium for transportation to the laboratory.

Aspirated Material—Material aspirated from abscesses or other lesions should be examined as soon as possible. Such specimens are received more frequently for diagnosis of extra-intestinal amebiasis than for other infections and should be expected to contain only the trophozoite stage of the ameba. Since this stage rapidly deteriorates, delays in examining the material may result in unreliable diagnoses. Demonstrating amebae in the thick pus removed from the abscesses is difficult for several reasons: 1) most of the organisms are in the peripheral area of the abscess and often relatively few are free in the abscess contents; 2) the organisms may be partially immobilized by coagulum; 3) since the fluid is sterile, amebae will not grow in culture when the material is inoculated in the manner routinely used for stool cultures. Streptodornase, a proteolytic enzyme, has been used in freeing the amebae from aspirates of liver abscesses (Freedman *et al.*, 1958). A suggested procedure for treating aspirated material is given in the Techniques section.

Aspirated specimens can be examined by the routine procedures used to demonstrate or recover trophozoites from feces. They may be examined in wet saline mounts and, if trophozoites are found, temporary stains such as Quensel's or buffered methylene blue may be used to confirm the findings. Permanent stains also can be made by any of the usual techniques for fecal preparations. If the aspirated material is liquid, it should be mixed with PVA-fixative which acts as an adhesive as well as a fixative. Difficulties in obtaining satisfactory smears may be encountered, however. Aspirated material can be cultured, if the medium is conditioned with *Clostridium perfringens.* (See section on Techniques.)

Biopsied Material—Biopsied material may be examined by several methods: sectioning and staining, compression, testing, and digestion. The method used will depend to some extent on the type of tissue and the organisms suspected.

In the diagnosis of trichinosis, muscle tissue may be examined by any of the techniques listed (Gould, 1945). The simplest and quickest methods, of course, are compression and teasing. However, digestion of the entire biopsy may reveal larvae when the infection is too light to be detected by direct examination. The digestion and compression techniques are given in the Techniques section.

Tissue to be examined for schistosome eggs may be compressed between two slides (Lichtenberg and Valladares, 1955) or cleared in lactophenol (1:1) and examined directly. The usual histologic methods of sectioning and staining can also be used. Tissue for examination for *E. histolytica* should be sectioned and stained. Juniper *et al.* (1958) have described an improved technique for handling punch biopsies from suspected amebiasis cases. It is described in the section on Techniques.

On occasion, liver biopsy may be done for the diagnosis of larva migrans infections. Histologic methods are most often used in preparing the specimen for examination. Serial sections should be examined.

SELECTION OF PROCEDURES FOR LABORATORY REGIMENS

Since many techniques and procedures have been advocated for the laboratory confirmation of intestinal parasites, some selection has to be made in establishing a laboratory regimen. Each type of examination has advantages and limitations and all are subject to technical errors. In general, the more types of examinations included in the laboratory routine, the greater the number of infections discovered. From a practical standpoint, however, the laboratory must decide if the increase in positive findings warrants the additional time and expense of performing the added techniques. In practice it would seem advisable to select certain procedures for routine use and to reserve others as supplementary techniques for special occasions. For routine purposes, the laboratory should include methods which will permit the recovery and identification of both trophozoites and cysts of intestinal protozoa and eggs and larvae of helminths. The accessibility of the patient and whether specimens are received fresh or through the mail will also influence the choice of techniques.

In most instances, examining preparations from a concentration procedure and permanently stained films should constitute the minimum for those laboratories attempting to render satisfactory service. The concentration procedure will help reveal light infections; the oil immersion examination of stained slides will reveal organisms overlooked at lower magnification and enable the microscopist to study critically the organisms encountered. In some circumstances, the preparation of direct wet mounts and the cultivation of stool specimens would be techniques of choice.

PROCEDURES FOR CENTRAL DIAGNOSTIC OR PUBLIC HEALTH LABORATORIES

FECAL SPECIMENS—Laboratories which are not associated with clinics and hospitals generally do not have the opportunity to examine fresh specimens. Central diagnostic laboratories, for example, those of State health departments, usually have to depend upon specimens delivered by carrier or by mail. These laboratories, therefore, should provide physicians using their services with shipping kits for properly submitting fecal specimens for parasitologic examinations.

As discussed in the section on collection, a two-vial kit (one vial containing 5 or 10 percent formalin, the other PVA-fixative) has been found satisfactory. In order to obtain full benefit from the preservatives, portions of the specimen should be put into the two vials immediately after passage and thoroughly mixed with the solutions. An illustrated direction sheet can be included with the kit to emphasize this point (Figure 5). As previously stated, an expiration date for using the PVA-fixative should be indicated. Two months is suggested as a practical time limit. When the specimen arrives at the laboratory, the formalin-ether concentration technique should be performed on the portion preserved in formalin and permanently stained films prepared with the portion preserved in PVA-fixative. The PVA-fixative material can be satisfactorily examined only by permanently stained preparations; wet mounts of this material are of no value. If time permits, direct wet mounts of the formalin preserved materials should be examined, since concentration techniques do not always reveal all organisms present.

The wet mounts are examined for cysts, eggs, and larvae, and the stained films are searched with the oil immersion objectives for trophozoites and cysts. A suggested procedure for handling fecal specimens is given in Figure 6.

OTHER SPECIMENS—The most common type of specimen submitted to central diagnostic or public health laboratories is feces, but occasionally anal swabs, vaginal exudates, aspirates, and other materials may be received. Suggested procedures for examining these materials are given in the following section.

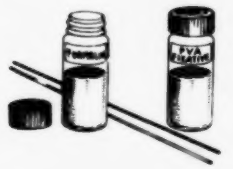
PROCEDURES FOR HOSPITAL AND CLINIC LABORATORIES

Laboratories in clinics and hospitals have advantages in the diagnosis of parasitic diseases over laboratories dependent on the mails for the delivery of specimens. Since fresh specimens can be obtained in clinic and hospital laboratories, direct wet mount examinations, concentrations, and permanently stained smears should

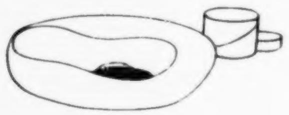
Figure 5.
PVA-Fixative for Submitting Stool Specimens to be Examined for Parasites

ADAPTED FROM BROOKE AND GOLDMAN, 1949

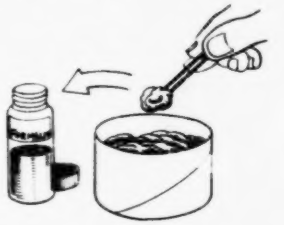
NOTE: BOTH SOFT AND *FORMED* SPECIMENS SHOULD BE SUBMITTED BY THIS METHOD.
SPECIMENS MUST BE *FRESH* WHEN PLACED IN VIALS.



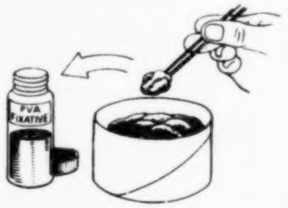
1 THE KIT CONSISTS OF TWO GLASS VIALS (ONE WITH 5% FORMALIN AND ONE WITH PVA-FIXATIVE) AND TWO APPLICATOR STICKS.



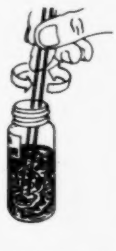
2 THE STOOL SHOULD BE PASSED INTO A DRY CONTAINER. URINE SHOULD NOT BE PASSED INTO THE SAME CONTAINER.



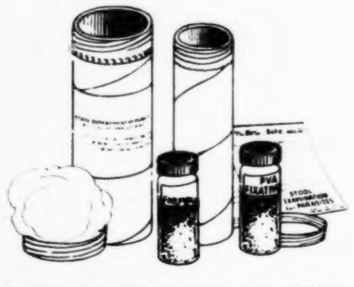
3 USING APPLICATOR STICKS, PLACE A QUANTITY OF THE STOOL (ABOUT THE DIAMETER OF A QUARTER) INTO THE VIAL CONTAINING FORMALIN. SCREW CAP ON TIGHTLY.



4 PLACE A SIMILAR QUANTITY INTO THE VIAL CONTAINING THE PVA-FIXATIVE.

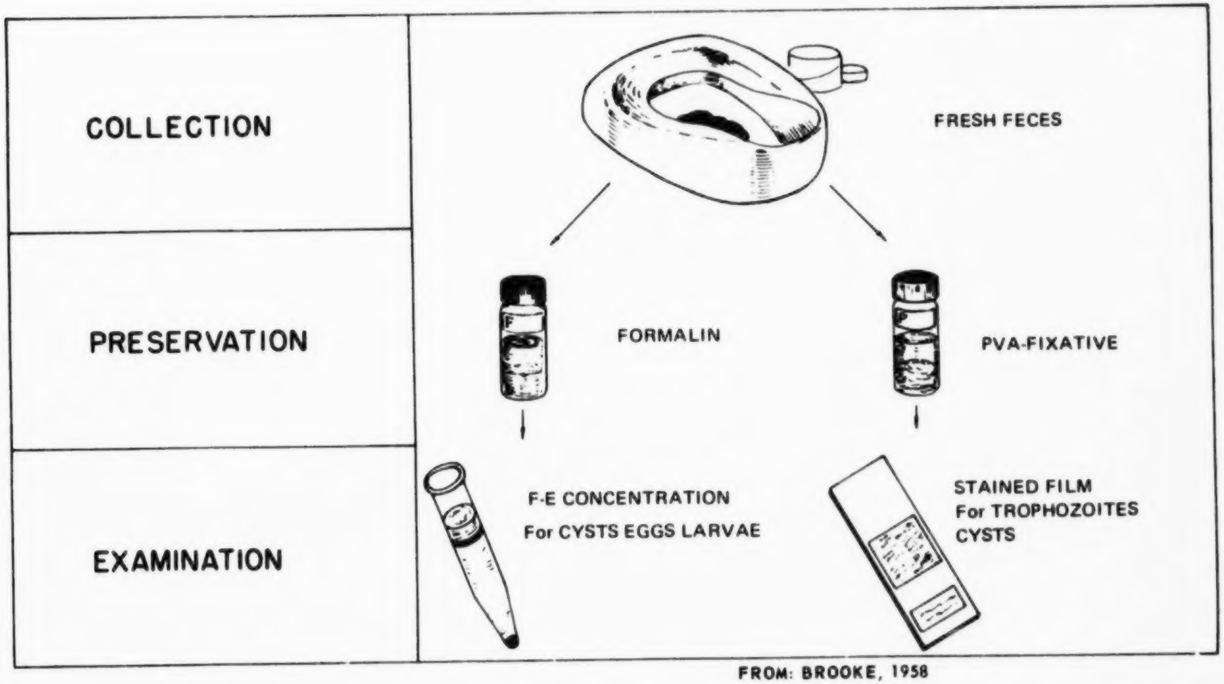


5 THOROUGHLY BREAK UP SPECIMEN IN THE PVA-FIXATIVE, USING APPLICATOR STICKS. SCREW CAP ON TIGHTLY AND SHAKE VIGOROUSLY.



6 PACK THE TWO VIALS SO AS TO PROTECT AGAINST BREAKAGE, ENCLOSE APPROPRIATE IDENTIFICATION, AND MAIL OR DELIVER TO YOUR PUBLIC HEALTH LABORATORY.

Figure 6.
Parasitological Examination of Fecal Specimens


COLLECTION
PRESERVATION
EXAMINATION
FRESH FECES
FORMALIN
PVA-FIXATIVE
F-E CONCENTRATION
For CYSTS EGGS LARVAE
STAINED FILM
For TROPHOZOITES
CYSTS

FROM: BROOKE, 1958

be used. In addition, cultivation may be of value in diagnosing protozoan infections (Norman and Brooke, 1955a). Furthermore, in the clinic or hospital, additional or purged specimens can frequently be obtained, and the patient is available for sigmoidoscopy, if necessary.

SPECIMENS FROM THE INTESTINAL TRACT

Normally Passed Fecal Specimens—Normally passed specimens should be delivered to the laboratory immediately. As soon as the specimen is received, it should be examined macroscopically. The consistency of the feces and any abnormalities present should be noted. If the specimen is soft, loose, or watery, it should be examined microscopically at once. Formed specimens may be left for a few hours without undue deterioration, but, for best results, they should be examined as soon as possible.

Fresh, unpreserved specimens should be examined routinely by direct wet mounts, by concentration methods, and insofar as possible, by permanently stained preparations. If time and facilities permit, cultivation may be advantageous, especially if rare trophozoites are found in the direct mount examination.

The following methods and sequence of examination are suggested as a routine procedure for parasitologic specimens.

1. Immediately upon receipt of the specimen, prepare one or two smears and place them in Schaudinn's fixative or, if preferred, emulsify a portion of the feces in PVA-fixative. Thus, smears for permanent staining will be available if and when they are needed. If necessary, smears can be left in Schaudinn's fixative for a day or two without harm.

2. Examine direct wet mounts, both unstained and stained. If mucus is present, include these portions in the feces selected for the preparations.

3. Perform either a formalin-ether sedimentation or a zinc sulfate flotation on all except watery specimens which would be expected to contain trophozoites but not cysts. In suspected cases of helminthiasis, however, concentrations should be performed on all types of specimens, because eggs and larvae may be present in watery as well as formed feces.

4. Stain and examine one of the fixed fecal smears for protozoa, especially if organisms suggestive of *E. histolytica* or questionable objects are observed in the wet mounts. Permanent stain preparations should also be examined if the specimen reveals any abnormalities (blood, mucus, or other material) or is of soft, loose, or watery consistency. The trichrome and chlorazol black stain techniques are especially useful for hospital and clinic laboratories. If chlorazol black is used, the smears are prepared and placed directly in the stain solution, since it is both fixative and stain combined. Ideally, permanent stains should be done routinely.

As stated above, cultivation may be advantageous, especially in suspected cases of amebiasis. It is not, however, recommended as a routine procedure. To obtain the full effectiveness of the procedure, the sediments of those cultures revealing trophozoites on wet mount examination must be stained (Norman and Brooke, 1955b). Mixing the culture sediment with PVA-fixative will facilitate the preparation of stained smears, since the PVA acts as an adhesive.

If no organisms or only unidentified organisms are reported in a specimen from a patient suspected of having a parasitic infection, especially amebiasis or giardiasis, additional specimens should be submitted for examination.

Postcathartic Specimens—In examining a series of postcathartic specimens, the first one passed should be concentrated by the formalin-ether technique, and all specimens in the series should be examined by temporary wet mounts. Permanently stained smears should be prepared from any of the specimens revealing protozoa or questionable structures. If neither the concentration nor any of the direct wet mount examinations is posiitve, a permanently stained smear should be prepared from at least one of the soft specimens. Since organisms may sometimes be found in mucus more easily than in non-mucoid material, flecks of mucus should be examined either as wet mounts or stained slides. If desired, one or more of the specimens can be cultured as a supplementary procedure. After purgation, it usually requires several days for any protozoan infection which may be present to develop to a demonstrable level. Therefore, there is little advantage in collecting additional stools, with or without catharsis, until a week has elapsed.

Sigmoidoscopic Materials—There should be no delay in the examination of material collected through the sigmoidoscope for diagnosis of amebiasis. If possible, the microscopic examinations should be made in the same or adjacent room in which the sigmoidoscopy is performed. Material which is aspirated or scraped from the mucosa should be mixed with saline and mounted. It is not necessary to use a warm stage, but ideally the room should be warm. After completing the direct examinations, the coverslip can be removed, the material mixed with 3 or more drops of PVA-fixative and dried and stained (Brooke, *et al.*, 1959.)

Combination of Methods—If there is sufficient time and clinical justification, combinations of the above examinations will increase the possibility of diagnosing cases of intestinal parasite infections, particularly amebiasis. For example, the clinic or hospital might decide to establish the following regimen: 1) examination of 3 normally passed stool specimens collected several days apart, 2) purgation with examination of 3 or more passages, and 3) sigmoidoscopy with examination of aspirated materials.

Since sigmoidoscopy reveals only a few inches of the colon and permits recovery of such small amounts of material, it should seldom be the sole method used.

OTHER TYPES OF SPECIMENS—For diagnosis of infections caused by certain species of intestinal parasites, materials other than feces will be received for examination. These materials should be examined as described under the heading "Examination Methods: Other Specimens" and in the following paragraphs.

Aspirated Material—Material aspirated from lesions is more often obtained in suspected cases of amebiasis than in other parasitic infections. It should be examined directly in wet mounts, using saline or temporary stains such as Quensel's or buffered methylene blue, for the presence of trophozoites or cultured, as previously described. If trophozoites or questionable structures are found, permanently stained slides of either fresh or PVA-fixed material should also be prepared.

Anal Swabs—For the diagnosis of enterobiasis, anal swabs should be collected. Either the cellulose tape slide preparation or the vaseline-paraffin swab is satisfactory. However, since it is best to collect eggs at night or in the early morning, the responsibility for taking the swab usually rests with the patient or, more commonly, with the parents of the patient, since children are the chief victims of this parasite. Explicit instructions on the proper techniques of collecting, handling, and transporting of the preparations should therefore be provided. An illustration of the cellulose tape method (Figure 11) has been successfully used in many cases. The person collecting the specimens should also be instructed to wash the hands thoroughly after each collection to avoid cross contamination of swabs from several patients. The patient should understand that several daily collections may be necessary.

Urine, Vaginal Swabs, Duodenal Drainage, and Sputum—The handling of urine, vaginal swabs, duodenal drainage and sputum has been discussed in the previous section. Selecting a technique for these body materials is not so much a problem as collecting and handling the specimen before examination. In all cases, the specimens should be examined as soon as possible or preserved in appropriate solutions: formalin for urine or sputum to be examined for helminth eggs or PVA-fixative for urine, sputum, vaginal material, or urethral exudate to be examined for protozoa.

PROCEDURES FOR SURVEYS

In surveys to determine the prevalence of intestinal parasites, the procedures used should recover all stages and types of parasites and at the same time be limited enough to be practical. The FE concentration technique and permanently stained films performed on fecal samples preserved respectively in formalin and PVA-fixative proved satisfactory in several surveys the National Communicable Disease Center conducted in cooperation with other organizations (Brooke *et al.*, 1955, 1956, 1958, 1962, 1963b; LeMaistre *et al.*, 1956; Melvin and Brooke, 1962).

The only part of the operation that has to be performed in the field is the preservation of the specimens as they are collected. Since unskilled personnel can do this with practically no equipment, the expense of the field aspects of the survey can be kept at a minimum. If necessary, however, various segments of the laboratory work can be conducted in the field. A preliminary estimate of the prevalance of infection can be obtained by performing the formalin-ether concentrations during the field investigations. The portions of the specimen preserved in PVA-fixative can be examined later in the central laboratory. On other occasions it may be preferable to a) concentrate the specimen and ship the sediments in formalin to the central laboratory for examination, and b) prepare the dried films of PVA-fixed specimens for staining and examining at a later time.

If the laboratory work involved in the survey must be reduced, the examination of the stained PVA films can be restricted to a) specimens which reveal protozoa or questionable organisms in the concentrations and b) specimens that were soft or contained blood or mucus when collected. The consistency indicated by the letters F, S, L, or W, the age of the specimen at the time of preservation, and any abnormalities (such as blood or mucus) should be written on the label affixed to the vials or otherwise properly recorded.

PROCEDURES FOR POST-TREATMENT SPECIMENS

The disappearance of symptoms in a patient with a parasitic infection is not sufficient evidence of a "cure." Post-treatment specimens should be examined to determine the effectiveness of therapy.

With most drugs used to treat patients with protozoan infections, organisms may not be found shortly after therapy even though the infection has not been eradicated. Thus, unless symptoms reappear earlier, the first post-treatment specimen should be collected approximately 4 weeks after therapy. Additional examinations after 3 and 6 months are advisable to detect later relapses.

In helminth infections, post-treatment examinations may be made after 1 to 2 weeks.

The specimens should be collected in the same manner as for the initial diagnosis, that is, three normally passed specimens should be collected at 2- to 3-day intervals. Although the patient can be requested to return to the clinic for another series of examinations, the specimens may be more conveniently and economically collected at the patient's home and shipped to the laboratory in formalin and PVA-fixative. Before the patient leaves the clinic he can be given 3 collection kits and instructed to submit specimens on specified dates.

The specimens can be examined as described previously for normally passed feces.

Post-treatment follow-up for enterobiasis should be made by examining anal swabs. Some workers recommend that the patient be examined for 7 days following completion of therapy and then periodically for 1 month. For practical purposes, 3 consecutive daily swabs might be examined 7 to 10 days after therapy and, if possible, a second series at the end of a month. Reinfection is common and, if swabs become positive after being negative for a month, the patient has probably reacquired the parasite.

REPORTING RESULTS

All parasites found, both pathogenic and non-pathogenic species, should be reported by their scientific names. All helminths are considered pathogenic or potentially so, but certain of the intestinal protozoa are commensals or non-pathogens. Although commensals have no clinical significance, they do indicate that the patient has ingested material contaminated with human feces. In persons harboring amebae, multiple infections have been found more frequently than can be accounted for by chance association (Boughton and Byrd, 1938; Eyles *et al.*, 1953). Finding a non-pathogenic species, therefore, increases the likelihood that the individual may be infected with a pathogen. Additional specimens should be examined.

In certain situations it might be advisable to identify the protozoa reported as pathogenic or non-pathogenic species.

Occasionally, protozoa, especially amebae, cannot be specifically identified. In these cases, an equivocal result should not be reported lest it be misinterpreted. A report of "organisms resembling *E. histolytica*," for example, may be interpreted as a final identification of this pathogen and may lead to needless treatment of the patient. Vague reports of "amebae found" may also be accepted as indicating the presence of *E. histolytica*. If species identification cannot be made, "unidentified amebae found," "unidentified flagellates found," or "unidentified nematode (or trematode, cestode or helminth) egg (or larva) found" should be reported. Additional specimens should be examined.

Although stages of helminths (eggs or larvae) are usually reported, stages of protozoa need not be reported. If cysts are found in the stool, trophozoites must be present in the intestinal tract.

If no organisms are found, the specimen should be reported as "no parasites found (NPF)" and not as "negative." If additional laboratory procedures had been used or if some other person had examined the specimens, organisms might possibly have been found.

PRESERVATION OF SPECIMENS

Feces and other body materials, unless examined very soon after passage, must be preserved for organisms to remain in a satisfactory, recognizable condition.

A single solution is not available for preserving all of the diagnostic forms (helminth eggs and larvae and protozoan cysts and trophozoites) and for providing material that can be both concentrated and permanently stained. Therefore, to permit reliable diagnoses, more than one preservative solution may be needed.

Some fixatives, such as merthiolate-iodine-formaldehyde (MIF) and phenol-alcohol-formaldehyde (PAF) preserve all four stages, but permanent stains cannot be prepared from these specimens. Since both MIF and PAF solutions involve temporary stains for wet mounts, they are discussed in the section dealing with wet mounts.

FORMALIN

While formalin is not the ideal preservative for protozoan cysts, it is satisfactory for collecting specimens to be examined for eggs, larvae, and cysts. In addition, it is simple to prepare, is stable, and does not require any "last-minute" mixing. Two concentrations are recommended: 5% for preserving specimens containing protozoa and 10% for specimens containing helminths. For routine purposes where both helminths and protozoa are sought, 5% is preferred. *Ascaris* eggs and sometimes *Trichuris* eggs, will continue to develop in 5% concentrations, but this does not interfere with identification of these species.

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PREPARATION

A. Formalin, 10%

Formalin (commercial formaldehyde)	10 ml
Water (tap or distilled)	90 ml

Mix thoroughly. Commercial formaldehyde is approximately 40% concentration by volume, which means that 10% formalin is actually about 4% formaldehyde.

B. Formalin, 5%

Formalin (commercial formaldehyde)	5 ml
Water (tap or distilled)	95 ml

Mix thoroughly.

TECHNIQUE FOR USING

Specimens for Routine Diagnosis—Mix 1 portion of feces in about 3 volumes of formalin. Since formalin is chiefly a preservative for cysts, eggs, and larvae, the more formed portions should be selected, and the softer parts saved for PVA fixation.

Teaching or Reference Specimens—If specimens are preserved for reference or teaching purposes, the following procedure is suggested.

1. Thoroughly comminute the fecal specimen with saline or water and strain through gauze or screens to remove coarse debris. Beakers or other suitable containers may be used, but a conical sedimentation flask is preferred.

2. Allow the specimen to settle for at least an hour.

3. Carefully pour off the supernatant fluid and preserve the sediment in 2 to 3 volumes of either 10% or 5% formalin. For protozoan cysts, helminth larvae, and certain eggs, such as *Hymenolepis diminuta* and *Hymenolepis nana*, 5% formalin is best. For other organisms, use 10%. At least 3 parts of formalin to 1 of fecal material should be used to insure adequate preservation.

4. If material is stored for future study, it should be checked frequently for evaporation of the formalin. Protozoan cysts and helminth larvae can be maintained in better condition if the preservative is changed at 6 month intervals. Addition of fresh formalin retards degeneration of the organism. Neutral formalin can be used, if preferred, and may give better long-term preservation.

5. Certain protozoa and helminths are more difficult to preserve than others and require greater care in handling. Among these are *Entamoeba histolytica* cysts, *H. nana* eggs, and rhabditiform larvae of *Strongyloides*. The other protozoa and helminth eggs preserve somewhat better and can be kept in good condition for longer periods.

PVA-FIXATIVE

The polyvinyl alcohol (PVA) fixative solution (Brooke and Goldman, 1949) furnishes a means of preserving trophozoites (and cysts) for subsequent examination and is particularly useful for shipping specimens to central laboratories. Fresh specimens, feces or other materials, suspected of containing trophozoites are mixed with PVA-fixative—a mixture of fixative and water-soluble resin. Immediately or months later, permanently stained films can be prepared from the preserved material. Trophozoites and cysts, if originally present, can be demonstrated by microscopic examination with oil immersion objectives. A distinct advantage of PVA-fixative is that it makes possible successful staining of organisms occurring in fluid specimens. The solution serves as an adhesive as well as a preservative and prevents loss of organisms during the staining procedure. By mixing the material with the fixative, diarrheic specimens and culture sediments can be stained satisfactorily. PVA-fixative is stable and can be kept for long periods in tightly stoppered bottles. Small quantities, however, as distributed for specimen collection, may harden or jell after a time. Therefore, a time limit for satisfactory use should be indicated on the collection vials to be sure of good preservation.

PVA powder, produced by E. I. DuPont de Nemours and Company, is designated Elvanol. In addition to the original grade, Elvanol 90-25, other grades of high hydrolysis, medium viscosity polyvinyl alcohol (71-24, 71-30) have proved satisfactory. PVA powder and PVA-fixative solution may be purchased from Delkote, Inc. (P.O. Box 1335, Wilmington, Delaware 19899). When ordering PVA powder, specify the pre-tested powder for use in PVA-fixative. PVA powder is also produced by Shawingan Resins Corporation (Springfield, Massachusetts 01101). This product is designated Gelvatol Resin. The grade 3-60 has been found satisfactory for use in the preparation of PVA-fixative.

PREPARATION

Saturated Aqueous Solution of Mercuric Chloride

Mercuric chloride crystals	130 to 140 gms
Distilled water	1,000 ml

Dissolve mercuric chloride in water by heating. Let cool. Excess mercuric chloride will crystallize out. Decant the clear solution or filter. Store in a glass-stoppered bottle. The exact amount of mercuric chloride needed to produce a saturated solution may vary but 130 to 140 gms usually will be sufficient.

Modified Schaudinn's Fixative

Glacial acetic acid	5.0 ml
Glycerol	1.5 ml
Schaudinn's fixative	93.5 ml

(2 parts saturated aqueous solution of mercuric chloride and 1 part of 95% ethyl alcohol)

PVA-fixative Solution

1. Slowly add 5 grams of polyvinyl alcohol (PVA) powder to 100 ml of modified Schaudinn's fixative *at room temperature, stirring constantly.*
2. Heat to about 75°C or higher until powder dissolves and suspension clears. Do not boil. A magnetic stirrer with a heating element (stirrer-hot plate) will facilitate preparation.
3. Cool to room temperature. The solution should be clear and free of lumps. Solutions prepared with some lots of PVA may remain turbid and exhibit some precipitate upon cooling. Unless these conditions are excessive, they will not interfere with satisfactory use of the solution. PVA-fixative remains satisfactory for several months and can be used either at room temperature or heated to 50°C.

Recently Burrows (1967b) recommended the following improved method of preparing PVA-fixative:

Modified Schaudinn's Fixative

Mercuric chloride crystals	4.5 gms
95% ethyl alcohol	31.0 ml
Glacial acetic acid	5.0 ml

1. Dissolve the mercuric chloride in the alcohol in a stoppered flask (50 or 125 ml size) by swirling at intervals.
2. Add the acetic acid, stopper, and mix by swirling. Set aside until needed.

PVA Mixture

Glycerol	1.5 ml
PVA powder	5.0 gms
Distilled water	62.5 ml

1. In a small beaker, add the glycerol to the PVA powder and mix thoroughly with a glass rod until all particles appear coated with the glycerol. Scrape the mixture into a 125 ml flask.
2. Add the distilled water, stopper and leave at room temperature for 3 hours to overnight. Swirl mixture occasionally to mix.

PVA-fixative Solution

1. Heat a water-bath (or large beaker of water) to 70° to 75°C. Adjust heat to maintain this temperature range.
2. Place the loosely stoppered flask containing the PVA mixture in the bath for about 10 minutes, swirling frequently.
3. When the PVA powder appears to be mostly dissolved, pour in the fixative solution, restopper, and swirl to mix.
4. Continue to swirl mixture in the bath for 2 to 3 minutes to dissolve the remainder of the PVA, to allow bubbles to escape, and to clear the solution.
5. Remove the flask from the water bath and let cool. Store the PVA-fixative in a screw-cap or glass-stoppered bottle.

PVA-fixative prepared by this method is "reliably adhesive, does not thicken with age" (Burrows, 1967b), and fixes amebae and other protozoa as well as the solutions prepared by the original method. Any laboratory experiencing difficulty in preparing satisfactory PVA-fixative solutions should try this method.

TECHNIQUE FOR USING—To obtain the full advantage of PVA-fixative as a preservative, specimens should be thoroughly mixed with the solution immediately after passage, before the organisms lose their characteristic morphology. Use approximately 3 parts of fixative to 1 part of feces or other material. Feces or other materials may be mixed with PVA-fixative on slides or in vials. Preservation is perhaps more commonly done in vials, especially in the collection of routine diagnostic specimens. Fixing material directly on the slide is appropriate when the quantity is small and/or when trophozoites have been found in direct saline mounts and require permanent staining for identification. Slide fixation is especially useful with the small amounts of material obtained by sigmoidoscopy or by aspiration. Such material can be examined directly in saline, the coverslip removed, and PVA-fixative added to the saline suspension and spread into a film for future staining.

If sufficient material is available, fixation in vials is preferable. Vials of PVA-fixed material are especially valuable if a number of slides are needed for teaching or reference purposes.

On Microscope Slides. A drop of dysenteric stool or other material is placed on a microscope slide and mixed with 3 drops of PVA-fixative. With an applicator stick, the mixture is then spread (*not* smeared like a blood film) over approximately one-third of the glass surface as described in the section on "Staining Procedures: Preparation of Smears" and as shown in Figure 10. Dried films remain satisfactory for staining for several months. For best results, however, they should be stained within 2 months after preparation.

In Vials. A quantity of specimen is thoroughly mixed in a vial containing 3 or more parts of PVA-fixative. Films for staining can be prepared immediately or months later. (See Section on "Staining Procedures: Preparation of Smears" and Figure 10). If the specimen in the vial jells, it can be liquefied by heating in a water bath before making the films. Unfortunately, this can be done only 1 to 2 times before the smears become unsatisfactory. If specimens re-jell after the second liquefication, they should be discarded.

Staining—PVA films are permeable to all commonly employed staining reagents The films are therefore handled in the same way as smears fixed in the conventional manner with Schaudinn's fixative. (See section on Staining Procedures.)

MICROSCOPY

A good microscope and lamp are essential for examining parasitologic specimens, since the identification of organisms is dependent on the observation of morphologic details. As discussed in the section on "Methods of Examination," the microscope should be equipped with at least 3 objectives: low power (16 mm), high dry (4 mm), and oil immersion (1.8 mm). If a separate lamp is used, ideally it should have a condensing system and some means of regulating light intensity.

ILLUMINATION AND ALIGNMENT

The microscopes and illuminators should be properly adjusted and aligned. To do this, place the lamp about 6 to 8 inches away and directly in front of the microscope. If the filters are removable, take these out of the holder and direct the beam of light onto the center of the microscope mirror. The flat side rather than the concave side of the mirror should be turned towards the light. The reason for using the flat side is demonstrated in Diagrams A and B of Figure 7 on the use of the microscope mirror and condenser. After the microscope and mirror have been aligned, the mirror is adjusted so that the object to be examined appears illuminated to the naked eye. The microscope field should be filled with light.

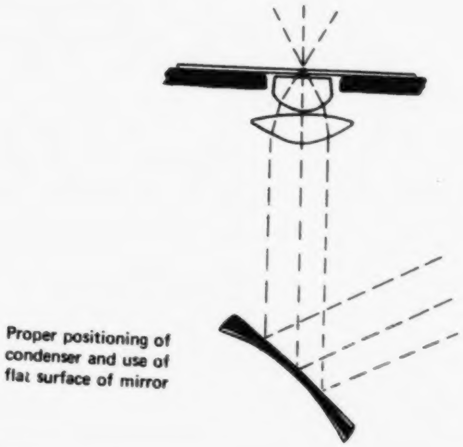
For optimal use the substage condenser should be in the upper range of the rack. Generally, with high dry, or oil immersion objectives, the condenser is moved to the top position. (See Diagram C on the chart.) Occasionally, with some microscopes, it may be necessary to lower the condenser slightly to fill the field with light when using the low power objective. The optimal position can be determined by removing the ocular and, with the substage iris diaphragm fully open, adjusting the condenser so that the back lens of the objective is filled with light.

CALIBRATION

Size is one of the important criteria used in identifying parasites or their diagnostic stages. A rough estimation can be obtained by

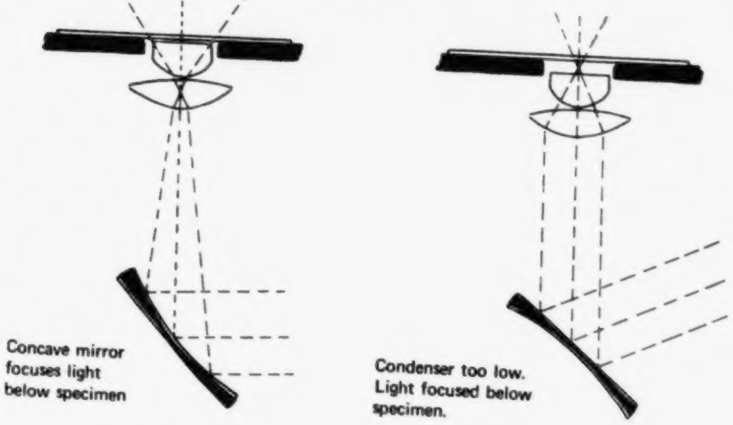
Figure 7.
Use of Microscope Mirror and Condenser

PROPER FOCUSSING



Proper positioning of condenser and use of flat surface of mirror

IMPROPER FOCUSSING



Concave mirror focuses light below specimen

Condenser too low. Light focused below specimen.

comparing the parasite with the size of human red blood cells (7.5μ to 8μ). However, if an organism is to be accurately measured, a calibrated micrometer disc that is placed within the ocular of the microscope should be used. The type most often used is a disc with a line scale divided into 50 spaces or ocular micrometer units.

Inasmuch as the units on the ocular micrometer are arbitrary and the exact value of each varies with different objectives and microscopes, the value must be calculated with each combination of lenses—low, high dry, and oil immersion. To do this, the ocular micrometer units are compared with a scale of known dimensions. This may be done by superimposing the image of the unknown ocular scale on the known scale of a stage micrometer (slide with a carefully etched and calibrated scale of 0.1 mm and 0.01 mm divisions) as demonstrated in Figure 8. The procedure for calibration is as follows:

1. Remove the 10X ocular from the microscope and unscrew the top eye-lens. Place the micrometer disc on the diaphragm within the ocular so that *the engraved side is underneath.* Screw back the eye-lens and insert the ocular in the microscope. Be careful to keep both micrometer and lens clean and free of dust which might interfere with observations.
2. Place the stage micrometer on the stage and focus on some portion of the scale.
3. Looking through the microscope, examine the ruling of the stage micrometer so that you can distinguish between the large (0.1 mm) and the small (0.01 mm) divisions.
4. Adjust the field so that the 0 line on the ocular micrometer is exactly *superimposed* upon the 0 line on the stage micrometer.
5. Without moving the stage micrometer find another point at the extreme right where two other lines are exactly superimposed. This second set of superimposed lines should be as far as possible from the 0 lines, but the distance will vary with the objective and microscope.
6. Knowing that each of the large divisions of the stage micrometer equals 0.1 mm, determine the total distance (in millimeters) between the two points of superimposition and the number of small ocular units necessary to cover the same distance.

   For example: Suppose 49 ocular units (small) equal 8 stage units (large) or 0.8 mm.

7. Calculate the number of millimeters that is measured by one small ocular unit.

   For example: 49 ocular units = 0.8 mm

   $$1 \text{ ocular unit} = \frac{0.8 \text{ mm}}{49} \text{ or } 0.0163 \text{ mm}$$

8. Since measurements of the protozoa and other small organisms or structures are usually given in microns rather than millimeters, the above determination must be converted to microns. Since 1 millimeter equals 1,000 microns, the millimeter determination multiplied by 1,000 will give the number of microns measured by 1 small ocular unit.

   For example: 0.0163 mm × 1,000 = 16.3 microns (μ)

9. Record the calibrations of the ocular micrometer obtained with the 10X oculars combined with each of the three objectives. The lines of the stage micrometer will increase in magnification while those of the ocular micrometer will remain the same. With high and oil magnifications, the thinner ocular micrometer line must be centered on the broader stage micrometer line for more accurate measurement.

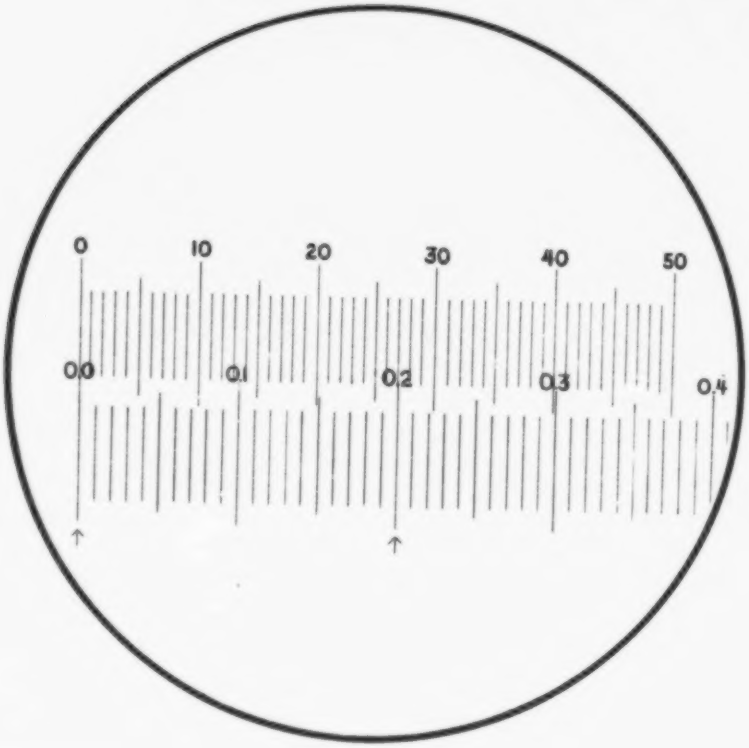
10. The size of any microscopic object can be quickly determined by measuring it with the ocular micrometer, noting the particular lens combination, and referring to the record of the ocular micrometer calibrations. For ready reference, the values for 1 to 50 units can be recorded in a chart such as the one below.

Ocular Micrometer Calibrations

Ocular magnification 10X

Low Power Obj. 16 mm		High Dry Obj. 4 mm.		Oil Immersion Obj. 1.8 mm.	
Units	Microns	Units	Microns	Units	Microns
1	16.3	1	3.5	1	1.7
2	32.6	2	7.0	2	3.4
3	48.9	3	10.5	3	5.1
4		4		4	
5		5		5	
6		6		6	
7		7		7	
8		8		8	
9		9		9	
10		10		10	

Figure 8.
Calibration of Ocular Micrometer


0
10
20
30
40
50
0.0
0.1
0.2
0.3
0.4

Ocular Micrometer - Top Scale
Stage Micrometer - Bottom Scale

DHEW, PHS, BDPEC, NCDC, LC&D

DIRECT WET MOUNTS

In addition to having good equipment, the microscopist should be proficient in preparing suitable mounts for examination and in examining these preparations.

PREPARATION

For direct wet mounts, as well as those from concentrated or cultured material, 3x2-inch slides, instead of 3x1-inch, should be used. The wide slide affords more space for preparation, permits the mount to be sealed around the edges, and prevents contamination of the microscope or the hands of the observer as might occur if the preparations "ran-over" on narrow slides. Ordinarily, a 22 mm-square coverslip of #1 thickness is used. For oil immersion examination, #2 thickness is too thick for focussing. Density of the mount is also important. The preparation should be thick enough that fine newsprint can just be read through it. If the mount is too thick, focussing will be difficult and the organisms may be obscured. If the mount is too thin, locating organisms takes longer and infections may be missed. Mounts should be prepared as follows:

FRESH UNPRESERVED FECES—Place a drop of physiologic saline (0.85%) on one end of the slide and a drop of iodine on the other end. Using an applicator stick or toothpick, pick up a very small portion of feces and emulsify it in each solution. Criteria for proper density should be kept in mind. Large particles should be avoided if possible, since the coverslip will not lie evenly on the slide. Cover the preparations, seal as described below, and examine. If too little fluid has been used and the material does not fill up the coverslip area, additional saline or iodine may be run under the edge. The feces will not be evenly mixed with the diluent, of course, but the preparation can be more easily examined than if vacant spots are present. On the other hand, the coverslip should not float on the fecal suspension. In this case, a piece of facial tissue or absorbent towel can be used to remove the excess before sealing. Iodine preparations will fade rather quickly if exposed to light, so these mounts

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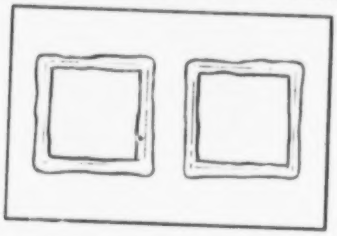
cannot be left indefinitely before examining. If several preparations are made at one time, a slide folder may be used to hold them.

SEALING OF PREPARATION—Sealing the mounts with a mixture of paraffin-vaseline (approximately 1:1), is recommended as a routine procedure to prevent drying, thus permitting reexamination or examination at a later time. Such preparations may last as long as 2 to 3 days. Sealed mounts prevent the "flow" of the material and can be more easily examined with oil immersion (as may be necessary to identify small protozoa) than unsealed preparations. Secure the coverslip by first placing a drop of hot paraffin-vaseline on the 4 corners of the mount. This will anchor the coverslip in place. A cotton swab or camel's hair brush can be used to apply the paraffin-vaseline. Next, spread a thin layer around the edges of the preparation (Figure 9). The layer of paraffin-vaseline should cover the edge of the coverslip, sealing it to the slide, but should not be very thick. If the layer is thick enough to form a ridge, the microscope objectives may become coated with the mixture as they are swung into position. Too much of the coverslip should not be covered with paraffin-vaseline, since a maximum surface of the preparation should be available for examination.

Figure 9.
Wet Mounts Sealed with Paraffin-Vaseline



FORMALIN PRESERVED FECES—If the quantity of formalin is more than 2 or 3 times that of the feces, discard the excess before resuspending the material. Place a small drop of iodine near one end of the slide. In preserved specimens, the formalin replaces the saline used in mounts of fresh feces, and the suspension can be used directly. Thoroughly mix the specimen before taking a sample, since eggs, larvae, and cysts settle to the bottom of the bottle. A drop of material from the top layers of an unmixed positive specimen may not contain organisms. Using 2 applicator sticks or toothpicks, held together, remove a drop of the suspension to the slide. If the suspension is thick, it may be necessary to add a small drop of saline to dilute it to a satisfactory density. Add a second portion to the iodine solution and mix. Cover both preparations and seal as described above. The same techniques used for fresh fecal mounts can be used to add additional fluid (saline can be added to the unstained mount) or to remove it. The preparation must be of the correct density to be of value.

In making mounts from preserved specimens, a small drop of iodine must be used so that the preparation will not be too thick. Since the iodine will be diluted by the formalin in the specimen, a slightly stronger concentration than usually used may be needed to obtain adequately stained structures. The need for a more concentrated iodine can be determined by trial. A too-strong solution, however, should be avoided. In the NCDC laboratory, Dobell's iodine is used routinely, and a darker, stronger solution is made up for use with formalinized specimens by adding extra iodine crystals to the potassium iodide solution.

CONCENTRATED SPECIMENS—Preparations from concentrated specimens are made in the same fashion as that described for formalin preserved material. Pipettes or loops, depending on the technique employed, are used to transfer material to the slides. Whether or not additional saline must be used will depend on how much fluid the sample contains. Often concentrated material, especially that from sedimentation procedures, will contain grit or large particles. Avoid getting these in the mount if possible, since, if this occurs, the coverslip will not lie evenly on the slide.

EXAMINATION

Helminth eggs and larvae are large enough to be seen under low power magnification (10X objective), although for study of structural details high dry magnification (43 or 44X objective) may be needed. Oil immersion is not necessary and, in fact, may tend to confuse the observer. Cysts and trophozoites of protozoa can be detected with the low power objective but higher magnifications are necessary for identifying species. Large protozoa, such as *Entamoeba coli* and *Balantidium coli*, and species like *Giardia lamblia* may be easily identified with high, dry magnification, but others, especially small cysts like *Endolimax nana* or *Entamoeba hartmanni*, may require oil immersion magnification for accurate identification. Diagnosis of *E. histolytica* cysts is also more reliable with oil immersion examination.

The light should be reduced for low power observations, since most organisms will be overlooked with bright light. Illumination should be regulated so that cellular elements in the feces are readily visible.

For routine examinations, a systematic scanning beginning with one corner of the mount and covering the entire preparation is recommended. This reduces the chances of missing scarce organisms that might be present. Scanning is done with the 10X objective. When organisms are found, the high dry and oil immersion objectives can be used for more detailed study. In the event no organisms are found, at least one complete saline (or unstained) mount should be searched carefully. An experienced microscopist usually requires from 10 to 15 minutes to do this.

The saline or unstained mount should be examined *first*, since eggs, larvae, trophozoites (if living), and cysts can be more readily detected in this type of mount than in the iodine preparation. If structures resembling cysts are seen, the iodine preparation should be examined. If no cysts or suggestive structures are found, the iodine mount need not be examined unless the microscopist wishes to do so. In certain instances, final species identification can be made; in others, only presumptive diagnosis will be possible.

In temporary wet mounts, eggs or larvae may sometimes need to be turned over or reoriented so that the structural details may be seen. This can be done by gently tapping the coverslip with an applicator stick. Occasionally an egg may be lying at an angle or in a different plane and appear unfamiliar. This is particularly true with *Trichuris* which may be oriented so that the viewer gets an optical cross-section or oblique view that may be unrecognizable as the familiar *Trichuris* egg. *Schistosoma mansoni* eggs also sometimes lie so that the spine does not show, and they too may need to be rolled over to be recognized.

IODINE SOLUTIONS

Several iodine solutions can be used to stain protozoan cysts satisfactorily. The two described below have been widely used and are simply prepared. The one recommended by Dobell and O'Connor (1921) is a weak iodine that should be prepared fresh about every 10 days for best results. Lugol's iodine, which must be diluted 1:5 for use, should be prepared fresh about every 3 weeks.

PREPARATION

1. Dobell and O'Connor's Iodine Solution:

Iodine (powdered crystals)	1 gm
Potassium iodide	2 gms
Distilled water	100 ml

Dissolve potassium iodide in the water. Add the iodine crystals and shake thoroughly. The crystals may not dissolve completely. Filter or decant. A portion should be put into a dropping bottle or other type of dispenser for daily use. The rest may be stored in a brown bottle away from the light. For practical purposes, when the iodine solution appears faded or when organisms are not staining properly, a few iodine crystals can be added and the solution shaken until the proper color is reached. Filter a portion into the dispenser bottle. Do not leave crystals in the solution as it may become too dark.

2. Lugol's Iodine Solution:

Iodine (powdered crystals)	5 gms
Potassium iodide	10 gms
Distilled water	100 ml

Dissolve the potassium iodide in the distilled water. Slowly add the iodine crystals and shake until dissolved. Filter. Store in a tightly stoppered, brown bottle away from the light. Dilute 1:5 with distilled water for use in staining protozoan cysts.

TECHNIQUE

A small portion of feces or fecal suspension is comminuted in a drop of the iodine solution, mounted with a coverslip, and sealed with a heated paraffin-vaseline mixture. The preparation should be of the correct density for microscopic examination.

In a correctly stained cyst, the glycogen, if present, appears reddish brown, the cytoplasm appears yellow, and the chromatin stains brown or black. The location of the karyosomes may be more easily determined and the character of the nucleus is more distinct in iodine preparations, but the chromatoid bodies are less visible than in saline mounts.

QUENSEL'S SOLUTION

The nuclei of amebae trophozoites are indistinct or entirely invisible in saline preparations. Iodine solution is unsatisfactory for staining this stage, since it may distort the entire organisms. Quensel's solution is recommended for the study of trophic amebae in temporary preparations (Svensson, 1935) and may be used with unpreserved feces or sediment from cultures.

PREPARATION

A. Stock Solutions

1. Sudan III Saturated Alcoholic Solution

Sudan III powder	1.6 gms
80% ethyl alcohol	100 ml

Add the stain to the alcohol. Shake thoroughly. Let stand for a few hours or overnight to be sure the solution will be saturated. If all of the stain dissolves, add more powder until no more will go into solution. Filter or decant to remove the excess powder and store in a screw-cap or glass-stoppered bottle.

2. Methylene Blue, Saturated Aqueous Solution

Methylene blue powder, medicinal	3.5 gms
Distilled water	100 ml

Add the stain to the water and shake thoroughly. Let stand for a few hours, shake at intervals. If all of the stain goes into solution, add more. Filter. Store in a screw-cap or glass-stoppered bottle.

3. Cadmium Chloride Solution

Cadmium chloride (c.p.)	10 gms
Distilled water	100 ml

Dissolve the cadmium chloride crystals in the water. Store in a screw-cap or glass-stoppered bottle.

B. Quensel's Stain Solution

Sudan III, saturated alcoholic solution	20 ml
Methylene blue (medicinal) saturated aqueous solution	30 ml
Cadmium chloride, 10% aqueous solution	50 ml

1. Mix the Sudan III and methylene blue.
2. Add the mixture to the cadmium chloride in an Erlenmeyer flask.
3. Gently shake the mixture now and then for 15 to 20 minutes. A voluminous flocculent precipitate develops and the fluid becomes almost colorless.
4. Filter. *Note:* The precipitate can be more easily removed from the paper (Step 7) if the material is filtered so that the residue (precipitate) is collected at the bottom of the filter cone rather than spread over a large area.
5. Remove all excess liquid from precipitate by placing the filter paper with the precipitate upon another filter paper or cellulose wool. Leave overnight.
6. Transfer precipitate to a fresh filter and rapidly pour through 25 to 30 ml of distilled water.
7. Dissolve the washed precipitate in 250 ml of distilled water.
8. Filter in a few days if fine crystals of cadmium chloride precipitate.

TECHNIQUE

A small amount of the fecal sample or culture sediment is picked up with an applicator stick, toothpick, or pipette (culture) and thoroughly comminuted in a drop of Quensel's solution. The mixture is covered with a coverslip and sealed. After about 10 to 20 minutes, the amebae trophozoites are stained a pale blue with their nuclei a deeper blue shade. The stained nuclei present the same morphologic characteristics that they do in a permanently stained hematoxylin preparation. Food inclusions within the cytoplasm are also stained. After ½ to 1 hour, the organisms become overstained and can no longer be identified. Occasionally, organisms fail to stain. The nuclei of *Dientamoeba fragilis* do not stain well, but the presence of 1 or 2 nuclei may be discernible. The use of a warm stage may aid in the staining of *Dientamoeba. Blastocystis hominis* stains beautifully, but ciliates, flagellates, and living cysts do not stain. Cysts preserved in formalin will stain, however, but details are not always easily seen.

BUFFERED METHYLENE BLUE

The pH of the stain solution has been found to be the deciding factor in bringing out the morphologic details of the nuclei of protozoa in wet mounts. Satisfactory results can be obtained by dissolving biological dyes, such as methylene blue, in an appropriately buffered solution. The buffered methylene blue stain can be substituted for Quensel's. For staining trophozoites of *E. histolytica* the exact pH is not critical and the optimum range may vary with the buffer system and dyes employed. Directions for preparing acetate buffers with the pH range of 3.6 to 4.8 are given below, or if desired, commercially available buffer tablets can be used. Phthalate buffers with a pH range of 2.0 to 2.5 are also satisfactory.

PREPARATION

1. Acetate Buffer Solution

Stock Solution A: 0.2 M solution of acetic acid (11.55 ml in 1,000 ml of distilled water)

Stock Solution B: 0.2 M solution of sodium acetate (16.4 gms of $NaC_2H_3O_2$ or 27.2 gms of $NaC_2H_3O_2 \cdot 3H_2O$ in 1,000 ml of distilled water)

Proportions of A and B for specific pH

Mix the indicated ml of A and B and dilute with distilled water to a total of 100 ml.

pH	A	B
3.6	46.3	3.7
3.8	44.0	6.0
4.0	41.0	9.0
4.2	36.8	13.2
4.4	30.5	19.5
4.6	25.5	24.5
4.8	20.0	30.0

2. Temporary Stain

Biological dyes like methylene blue or pyronin may be used. The quantity of dye that should be dissolved in the buffer solutions can be determined by a little experience. The amount will depend upon the solubility of the dye in the buffer at different pH levels. In practice a small quantity of methylene blue (0.06%) in acetate buffer at pH 3.6 has given satisfactory results.

TECHNIQUE

A small amount of the fecal sample or culture sediment is picked up with an applicator stick, toothpicks, or pipette and thoroughly comminuted in a drop of the buffered methylene blue solution. The mixture is covered with a coverslip and sealed. After a few minutes the amebae trophozoites are stained a pale blue with their nuclei a deeper blue shade. The stained nuclei present the same morphologic characteristics that they do in a permanently stained hematoxylin preparation. Food inclusions within the cytoplasm are also stained. The nuclei of *D. fragilis* stain with this solution and the presence of 1 or 2 nuclei may be discernible. Occasionally, some organisms fail to stain.

MERTHIOLATE-IODINE-FORMALDEHYDE (MIF) SOLUTION

The MIF solution can be used for staining protozoan cysts and trophozoites in wet mount preparations and as a preservative for these stages and helminth eggs and larvae (Sapero and Lawless, 1953). Slightly different formulas are recommended for these two uses.

PREPARATION

1. Wet Mount Preparations

Lugol's solution (see below)	0.10 ml
Formaldehyde (USP)	0.15 ml
Tincture merthiolate, No. 99, 1:1,000 (Lilly)*	0.75 ml

One ml of stain is sufficient for 25 to 30 fecal preparations. The stain is prepared fresh each day. The amounts of the 3 ingredients may be varied for different purposes. The greater the iodine strength the more immediate the staining reaction; however, the cysts and trophozoites are more easily detected at a lower iodine strength. Cytoplasmic detail is more distinct at a greater formaldehyde strength.

2. Preservation of Specimens

A. Stock "MF" Solution (Stable)

Distilled water	50 ml
Formaldehyde (USP)	5 ml
Tincture of merthiolate (1:1,000)*	40 ml
Glycerin	1 ml

Store in brown bottle.

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*Tincture of Merthiolate, No. 99, 1:1,000, (Lilly) (Eli Lilly & Co., Indianapolis, Indiana) must be used, since this contains eosin, the stain ingredient in the second stage.

B. Lugol's Iodine Solution (Prepared Fresh Every 3 Weeks)

Iodine crystals (powdered)	5 gms
Potassium iodide	10 gms
Distilled water	100 ml

Dissolve the potassium iodide in the distilled water. Add the iodine crystals slowly and shake until dissolved. Filter. Store in a brown bottle.

Mix solutions a and b together as indicated below to prepare the MIF preservative.

TECHNIQUE

1. Wet Mounts

a. Place 1 drop of distilled water on a slide using a standard medicine dropper.

b. Add 1 drop of MIF stain.

c. Comminute a portion of the fecal specimen in this mixture. Have the suspension of the correct density for a satisfactory mount.

d. Mount with coverslip, seal, and examine. The preparation may be examined several hours later without loss of staining or details.

2. Preservation of Specimens

a. Add 2.35 ml MF stock to 0.15 ml Lugol's solution *immediately before use* because prior addition of the iodine causes a dense precipitate to be formed and the iodine fails to act satisfactorily on the protozoa.

b. Thoroughly mix a portion of the feces about the size of a pea in the MIF solution (approximately 1 part of feces to 2 or 3 parts of preservative).
Do not use too much feces. For larger samples, use increased amounts of the MIF stain in the same proportions of MF stock and iodine solutions.

c. To examine, take a drop of fluid from the top layer of the sedimented feces and place on a slide. A preparation roughly comparable in density to a direct smear should be obtained.

STAIN REACTIONS

The staining reaction in both direct smears and preserved specimens is divided into two phases: 1) an iodine phase in which cysts and trophozoites stain a yellow-green or yellow-brown color, and 2) an eosin phase which replaces the iodine phase and is permanent.

In the iodine phase, nuclei stain dark brown and in the eosin phase, dark red to jet black. Cytoplasm of the trophozoites and cysts changes from yellow-green or brown in iodine to an eosin (pink or red) color in the second stage. Occasionally, refractory mature cysts are encountered which fail to stain in the eosin phase. Nuclear elements of all species except *D. fragilis* are fairly well defined. Glycogen appears as a dark brown area in the iodine stage and as a colorless area in the eosin stage. Chromatoid bodies are characteristic in appearance. Flagella may be detected on flagellate trophozoites. Helminth eggs also stain and retain their normal characteristics. Fecal debris stains darker brown than parasites or blood and tissue cells. No distortion occurs, but for good contrast, a blue filter should be used in the illumination system.

PHENOL-ALCOHOL-FORMALDEHYDE (PAF) SOLUTION

This fixative solution may be substituted for formalin in collecting fecal specimens (Burrows, 1967a). It preserves protozoa, including trophozoites, as well as helminth eggs and larvae. Protozoan nuclei are more distinct than with plain formalin. PAF-fixed material may be examined as unstained mounts or stained with thionin or azure A stains.

PREPARATION

1. PAF-Fixative

Phenol crystals (white, not old and discolored)	20 gms
Physiologic saline (0.85%)	825 ml

Dissolve crystals in saline, then add:

95% ethyl alcohol	125 ml
Formaldehyde	50 ml

Mix well. If liquefied phenol is available, 23 ml may be used in place of 20 gms of crystals.

2. Thionin Stain

Thionin powder	10 mg
Distilled water	100 ml

Dissolve powder in water. This stain is preferred for PAF-fixed specimens.

3. Azure A (or Methylene Azure A) stain

Azure A powder	10 mg

Dissolve powder in water.

TECHNIQUE

Preservation

Mix a portion of feces with the PAF-fixative solution using about 3 parts of solution to 1 part of feces. The feces should be emulsified in the solution as much as possible.

Examination

1. In the laboratory, allow the bottle containing the specimen to stand until the contents are well settled. Pour off about half of the excess fixative.
2. Mix the sediment with the remaining solution.
3. With a pipette, place a drop of the suspension on a 3×2-inch slide.
4. Add a drop of stain, preferably thionin stain, and mix thoroughly.
5. Cover with a 22 mm-square, #1 thickness coverslip, and seal. The preparation should be of a satisfactory density for examination.

STAIN REACTIONS

In this fixative, amebae trophozoites do not round up, but tend to be fixed with pseudopodia still extended. Cytoplasm of cysts and trophozoites stains light blue with slightly darker blue nuclei and chromatoid bodies. The individual granules of the peripheral chromatin of the nuclei are often visible. Although eggs are also stained, the staining is light enough not to obscure characteristics.

If the thionin stain appears to stain protozoa too heavily, dilute with distilled water. If the staining is too light, add a minute portion of stain powder (the amount that can be picked up on the end of a toothpick) to the solution.

CONCENTRATION PROCEDURES

FLOTATION

MODIFIED ZINC SULFATE CENTRIFUGAL FLOTATION TECHNIQUE

The zinc sulfate centrifugal flotation procedure was the first concentration technique developed for the recovery of both helminth eggs and larvae *and* protozoan cysts. Operculated eggs and those of the schistosomes are not recovered, however. The use of water for preparing the fecal suspension will destroy *Blastocystis* and remove some of the debris usually present. The original method developed by Faust *et al.* (1938) included 2 steps which are omitted in the modified procedure described below. These were 1) preparation of a fecal-water suspension in a beaker, and 2) straining a portion of the suspension through gauze into a small tube. In addition, unless the specimen is very oily, the number of washings by centrifugation is reduced to one.

Organisms which rise to the surface of the solution will begin to sink after about 1 hour. Therefore, for optimum recovery, slide mounts should be prepared as soon as the technique is completed. Since prolonged exposure to zinc sulfate may cause small cysts to distort and become difficult to recognize, mounts should not be allowed to stand for several hours before being examined.

Preparation

1. Add 331 gms of zinc sulfate (USP) to 1 liter of warm tap water. A technical grade of zinc sulfate can also be used if the insoluble salts are removed from the solution by filtering before use.
2. After the zinc sulfate is thoroughly dissolved, check the specific gravity with a hydrometer. It should read 1.18. If the reading is not correct, adjust the specific gravity by adding water or zinc sulfate as needed.
3. Some workers prefer a specific gravity of 1.20. This is prepared by using a slightly larger amount of zinc sulfate. This solution of higher specific gravity should always be used when a formalinized fecal specimen is to be examined.

Technique

1. Using 2 applicators, comminute a fecal sample about the size of a small pea in a 100×13 mm tube half filled with tap water. Make certain that all obvious particles are broken up and that an even suspension is formed.
2. Add additional tap water until the tube is two-thirds full.
3. Centrifuge this preparation for 1 minute at approximately 2,500 r.p.m.
4. Pour off the supernatant fluid into a container holding a disinfectant, for example, cresol or amphyll.
5. Repeat this washing only if the stool is extremely oily.
6. Add enough zinc sulfate solution to fill the tube half full.
7. Using an applicator, break up the packed sediment very thoroughly and suspend it in the solution.
8. Add additional zinc sulfate solution to fill the tube to within ½ inch of the top.
9. Centrifuge this suspension for 1 minute at 2,500 r.p.m.
10. *Without shaking or spilling*, carefully place the tube in a rack.
11. The material in the surface layers can be removed in either of two ways: a) by raising the fluid level to form a meniscus on top or b) by removing a portion of the surface film with a wire loop or pipette.

A. Raising Fluid Level

1) Slowly fill the tube to the brim with zinc sulfate *without allowing any runover.* Using a pipette or dropper, *add the solution down the side of the tube* to prevent disturbing the surface film.
2) Place a clean, grease-free #1 coverslip (22×22 mm) on top of the tube so that the under surface touches the meniscus. Leave undisturbed for about 10 minutes. No bubbles of air should be present.
3) Deftly remove the coverslip with a straight, upward motion. A drop containing eggs, larvae, and cysts (if present) will adhere to the underside of the coverslip.
4) Lower this onto a drop of iodine stain placed on a clean 3×2-inch slide. Seal the preparation.

B. Removing Surface Film

1) Make a small wire loop so that the loop is at right angles to the stem.
2) Slide the loop gently under the surface film and remove 2 or 3 loopfuls of material to a 3×2-inch slide.

3) A capillary pipette may also be used to transfer a portion of this film to a slide.
4) Add a drop of iodine, cover with a coverslip, and seal the preparation.

12. Examine for eggs, larvae, and cysts.

CONCENTRATED BRINE FLOTATION

This technique (Willis, 1921) is simple and efficient for the recovery of helminth eggs other than operculate and schistosome eggs. Protozoan cysts, however, will be unrecognizably shrunken. It is especially recommended for recovery of hookworm eggs.

Preparation

Prepare the brine solution by stirring sodium chloride (table salt is satisfactory) into hot or boiling tap water until the excess added will not go into solution. This saturated solution should be checked with a hydrometer after cooling to insure that the specific gravity is at least 1.20. The solution should be filtered before using.

Technique

1. Place a sample of feces about the size of a small marble into a vial about 1 inch in diameter and 2 to 2½ inches high, with a capacity of approximately 20 ml.
2. Fill this ¼ full with the saturated salt solution.
3. Using 2 applicators, mix thoroughly, suspending the feces as a fine silt.
4. Fill the vial to the brim with salt solution.
5. Carefully superimpose a grease-free 3×2-inch slide. The slide must contact the surface of the suspension completely without any airpockets being formed and without overflow.
6. Allow the preparation to stand for 10 minutes to 1 hour. The optimum time is 10 to 15 minutes.
7. Lift the slide straight up without tipping. A drop containing the eggs will adhere to the undersurface of the slide.
8. Invert the slide without allowing the drop to slide off. A coverslip is not necessary.
9. Examine for eggs immediately, before the preparation begins to dry.

SEDIMENTATION

GRAVITY SEDIMENTATION—One of the simplest techniques applicable to the recovery of all eggs, larvae, and cysts is a combination of staining and sedimentation. This method is especially suitable for operculate and schistosome eggs. Since the technique is essentially a washing process, concentration is often not marked and the preparation usually contains much confusing debris. The use of a large fecal sample in contrast to the smaller ones used in other techniques is of great help, especially when only small numbers of eggs are present.

Technique

1. Place a sample of feces about the size of a large marble (approximately 5 gms) in a 250 ml beaker, fill it one-fourth full with tap water, and mix thoroughly, using a tongue depressor or 2 applicators. Faust and Ingalls (1946) recommend the use of a 0.5% solution of glycerin in tap water as a substitute for plain tap water as an aid in the reduction of surface tension, thereby increasing the yield of organisms.
2. Add sufficient tap water to fill the beaker three-fourths full.
3. Strain this suspension through a funnel containing 2 layers of *wet* cheesecloth into a sedimentation glass or cone-shaped graduate (250 ml or 500 ml size).
4. Allow the suspension to settle for 1 hour.
5. Carefully pour off two-thirds of the supernatant fluid without allowing any of the sediment to escape.
6. Add fresh tap water near the top of the graduate so that the sediment becomes uniformly resuspended and let settle for another hour.
7. This washing procedure may be repeated until the supernatant fluid is relatively clear. Generally one washing will suffice. Schistosome eggs hatch within a short time after the feces are diluted with water. Therefore, if schistosome eggs are being sought, allow the suspension to settle only once; do not wash. Saline can be substituted for the water to retard or prevent hatching.
8. Carefully pour off the last wash water without losing any of the sediment.
9. Insert the tip of a long capillary pipette with rubber bulb attached into the *top* layer of the sediment and remove a portion to a 3×2-inch slide. If the preparation is too thick, dilute with a drop of saline. Iodine preparations should also be made if cysts are present, or suspected.
10. Cover with a coverslip and examine.
11. Take additional samplings from the middle and bottom layers of sediment.

CENTRIFUGAL SEDIMENTATION—This technique is essentially the same process as the gravity sedimentation, except that centrifugation concentrates the material more quickly. The technique has been found to be moderately effective for recovering eggs (particularly of *Schistosoma japonicum* and *S. mansoni*), larvae and cysts. It is rapid, reveals the organisms in a living condition, does not require expensive reagents to perform, and samples a large quantity of feces. Like the gravity sedimentation, however, the effective concentration is not marked and confusing debris is usually present. Centrifugation is the recommended procedure for concentrating urine for the recovery of *Schistosoma haematobium* eggs.

Technique

1. **Feces**

   a. Place a portion of feces about the size of a walnut (4 to 5 gms) in a 250 ml beaker. Fill the beaker one-fourth full with tap water and mix thoroughly with a tongue depressor to obtain an even fecal suspension. Break up any lumps or fecal masses.

   The fecal suspension can be prepared in a tube instead of a beaker if it is more convenient. Place the feces in a large test tube (25×150 mm), fill about one-half full with water, and mix thoroughly. If the feces is soft, it can be mixed with the water by stoppering the tube and shaking it for at least 1 minute.

   Physiological saline may be substituted for water to prevent hatching of schistosome eggs that might be present.

   b. Strain the suspension through a funnel containing 2 layers of wet cheesecloth or gauze into a 50 ml centrifuge tube.

   c. Centrifuge at 1,000 to 1,500 r.p.m. for 1 minute. Do not exceed 1,500 r.p.m. since some types of eggs may collapse at higher speeds.

   d. Carefully pour off the supernatant into a suitable disinfectant solution, without disturbing the sediment.

   e. Using a long capillary pipette with a rubber bulb attached, remove a portion of the top layer of sediment to a 3×2-inch slide. If the preparation is too thick, dilute with a small drop of saline. If cysts are present or suspected, iodine preparations should also be made. Cover the samples with a 22 mm-square coverslip, seal, and examine as directed for wet mounts.

   f. Remove a sample from the *bottom* layer and handle in the same manner.

2. **Urine**
   a. Fill a 15 ml centrifuge tube three-fourths full with the urine specimen.
   b. Centrifuge at 1,000 to 1,500 r.p.m. for 1 minute.
   c. Carefully pour off the supernatant fluid.
   d. Using a long pipette with rubber bulb attached, remove a portion of sediment to a 3×2-inch slide, cover with a cover-slip, and seal.
   e. Examine for eggs under low power; confirm under high, dry. *T. vaginalis* trophozoites may also be recovered by this method.

ACID-ETHER—This method is useful for concentrating most helminth eggs, especially for those of *S. mansoni* and *S. japonicum*. It is not satisfactory for the diagnosis of protozoan cysts.

The procedure, originally developed by Telemann (1908) relies upon the use of an acid, for example, HCL, to dissolve albuminous matter, such as casein, soaps, mucin, phosphates, and various calcium salts. Ether is then added to dissolve neutral fats and free fatty acids. These actions plus mechanical agitation result in the liberation of eggs from the debris in which they are buried.

The disadvantage of the technique is that the embryos in the eggs recovered are dead. Thus, in attempting to evaluate treatment for schistosomiasis, one cannot tell whether live or dead eggs are being passed. In addition, when used in routine work, ether is a relatively expensive reagent. However, technical grade is satisfactory.

Preparation of Hydrochloride Acid Solution

Concentrated hydrochloric acid	40 ml
Distilled water	60 ml

This results in a 15% concentration of hydrochloric acid.

Technique

1. Place a sample of feces about the size of a large pea or bean (approximately 1 gm) in a test tube or beaker containing 5 to 10 ml of the 15% hydrochloric acid.
2. Comminute *thoroughly* with applicator sticks to form a homogeneous suspension.
3. Strain 5 ml of the suspension through 2 layers of wet cheesecloth or gauze into a 15 ml, conical centrifuge tube, preferably graduated.

4. Add an equal quantity of ether; insert a rubber stopper and shake thoroughly for about 1 minute, keeping the thumb on the stopper. Loosen the stopper at least once to release the pressure.
5. Remove the stopper, place the tube in the centrifuge, and spin at 1,500 r.p.m. for 1 minute.
6. Four layers will appear in the tube: 1) a top ether layer, 2) a plug of fecal debris, 3) an acid layer, and 4) a bottom sediment containing eggs and larvae, if present.
7. Loosen the plug of debris by holding the tube horizontally and inserting an applicator stick between the plug and the wall of the tube in a ringing motion.
8. Quickly, *but carefully*, pour off the ether, plug, and acid layers, leaving the sediment undisturbed.
9. Do *not* rotate the tube back to a vertical position; keep it horizontal. This will prevent the remains of the fatty extract and debris from flowing down the side of the tube into the sediment. If the tube must be left in an upright position for a time, a cotton swab can be used to remove debris adhering to the glass.
10. Maintaining the tube in this position, use a capillary pipette with a rubber bulb attached to remove a portion of the sediment to a 3×2-inch slide. The sediment should be stirred before taking the sample.
11. Place a coverslip on the drop and examine under low power. If necessary, confirm findings under high dry.

Occasionally eggs are trapped in the layer of debris and, consequently, are not recovered in the sediment. To reduce the adhesive forces and increase settling of the eggs, 0.6 ml of a 10% aqueous solution of Triton NE* can be mixed with the acid-feces mixture (Weller and Dammin, 1945) in Step 1.

FORMALIN-ETHER—This concentration procedure is efficient in recovering protozoan cysts and helminth eggs and larvae, including operculated and schistosome eggs (Ritchie, 1948). Less distortion of cysts occurs with this technique than with the zinc sulfate method, and it is more effective in concentrating formalinized specimens. *H. nana* eggs may be missed however, and concentration of *Giardia lamblia* cysts and *Iodamoeba bütschlii* cysts may not be very good.

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*A 3% solution of Triton X-100 may be substituted for the Triton NE (33% concentration of Triton X-100). Triton X-100 is available from Rohm and Haas Co., Philadelphia, Pa.

Technique for Fresh Specimens

Note: If permanently stained slides are to be made, the smears should be prepared before the specimen is used for concentration. If a portion of the specimen is to be preserved in PVA-fixative, it should be done before proceeding with the concentration procedure.

1. Comminute a portion of stool about the size of a medium sized walnut in sufficient saline so that 10 ml of suspension will yield about 1 ml of sediment upon centrifugation. The suspension can be prepared in the carton in which it is submitted or in a beaker or flat-bottom paper cup. Water may be used in place of saline.
2. Strain about 10 ml of the suspension through a small funnel containing 1 or 2 layers of *wet* gauze into a 15 ml, conical centrifuge tube. If wide mesh cheesecloth is used, use 2 layers; if medium or narrow mesh gauze is used, 1 layer is sufficient. To conserve glassware, a cone-shaped paper cup (about a 4 ounce size) with the point cut off can be substituted for the funnel.
3. Centrifuge at 2,000 to 2,500 r.p.m. for 1 minute. Decant supernatant. About 1 to 1½ ml of sediment should be present. If the amount is much larger or smaller, adjust to the proper quantity in the following manner.
   a) Amount too large.
      Resuspend the sediment in saline (or water) and pour out a portion. For example, if the amount is twice the desired quantity, pour out about half (or slightly less) of the suspension. Add saline (or water) to bring the fluid level to about 10 ml and centrifuge again.
   b) Amount too small.
      Pour off the supernatant and strain a second portion of the original fecal suspension into a tube. The amount to be strained can be determined from the amount of sediment; that is, if about half of the quantity necessary is obtained with the first centrifugation, strain another 10 ml into the tube. Centrifuge again.

   It is not necessary to have an *exact* quantity of sediment in the tube, but the quantity should approximate the amounts indicated above. Too much or too little sediment will result in an ineffective concentration.
4. Resuspend the sediment in fresh saline (or water), centrifuge, and decant as before. This step may be repeated if a cleaner sediment is desired.

5. Add about 10 ml of 10% formalin to the sediment, mix thoroughly, and allow to stand for 5 minutes. At this point, the formalin-feces mixture may be stoppered and saved until a later time.

Note: Plastic squeeze bottles for saline and formalin will facilitate dispensing these solutions into the tubes.

6. Add 3 to 5 ml of ether, stopper the tube, and shake vigorously for at least 30 seconds. Remove the stopper with care.
7. Centrifuge at 1,500 r.p.m. for 1 minute. Four layers should result as follows: 1) layer of ether, 2) plug of debris, 3) layer of formalin and 4) sediment.
8. Free the plug of debris from the sides of the tube by ringing with an applicator stick and carefully decant the top 3 layers. Use a cotton swab to clean debris from the walls of the tube and prevent it from settling down into the sediment.
9. Mix the remaining sediment with the small amount of fluid that drains back from the sides of the tube and prepare iodine and unstained mounts in the usual manner for microscopic examination. If not enough fluid is left in the tube, a drop of saline can be added to the sediment.
10. If examination of the specimen is delayed, add 1 or 2 ml of 10% formalin to the sediment and stopper the tube. Formalinized sediments may be kept for some time if they do not dry. Remove the excess formalin before making the mounts, otherwise the specimen may be too thin for satisfactory preparations.

Technique for Formalin Preserved Specimens

1. Thoroughly stir the formalinized specimen.
2. Depending on the size and density of the specimen, strain a sufficient quantity through wet gauze into a conical 15 ml centrifuge tube to give the desired amount of sediment indicated below.
3. Add tap water, mix thoroughly, and centrifuge at 2,000 to 2,500 r.p.m. for 1 minute.
4. Decant supernatant and, if desired, wash again with tap water. The amount of sediment should be about ½ to ¾ ml. If too much or too little is present, adjust the quantity by the same methods described in step 3 for fresh material. In formalin-preserved specimens, the formalin has already clarified the feces to some extent and further clarification is caused primarily by the ether. Therefore, the reduction of the sample (or sediment)

is not as great as with fresh feces, and the initial quantity must be less.

5. Add 10 ml of 10% formalin to the sediment and mix thoroughly.
6. Add 3 to 5 ml of ether, stopper the tube, and shake vigorously for at least 30 seconds. Remove the stopper with care.
7. Centrifuge at 1,500 r.p.m. for about 1 minute. Four layers should result as stated above (Step 7).
8. Free the plug of debris from the sides of the tube by ringing with an applicator stick, and carefully decant the top 3 layers. Use a cotton swab to remove debris adhering to the glass.
9. Mix the remaining sediment with the small amount of fluid that drains back from the sides of the tube (or add a drop of saline, if necessary) and prepare iodine and unstained mounts in the usual manner for microscopic examination. After decanting the upper 3 layers (Step 8), a small amount of formalin may be added to the sediment, the tube stoppered, and mounts prepared later.

MERTHIOLATE-IODINE-FORMALDEHYDE (MIFC)—Specimens preserved in MIF solution may be concentrated by either gravity sedimentation or the MIFC sedimentation technique. The latter procedure is similar to the formalin-ether concentration in that it recovers both protozoan cysts and helminth eggs and larvae (Blagg *et al.*, 1955). The technique is the same as for the FE except that the formalin is replaced by MIF solution. (See directions for the use of MIF as a preservative.)

1. Sedimentation

A certain amount of concentration will occur if the specimen is allowed to settle normally. Since eggs, cysts, and trophozoites tend to collect in the upper layers of the sediment, mounts for examination should be prepared from this area.

Sedimentation can be speeded up by centrifuging the specimen (or a portion of it) at about 2,000 to 2,500 r.p.m. for 1 minute.

2. MIFC

a. Thoroughly mix the MIF fecal suspension and strain into a 15 ml centrifuge tube. If the quantity of specimen is insufficient, add fresh MIF preservative to make 10 ml.
b. Add 3 ml of ether. Stopper with a rubber stopper and shake thoroughly for at least 1 minute. Remove the stopper with care.

c. Centrifuge at 1,500 r.p.m. for 1 minute. Four layers will result: 1) ether, 2) plug of debris, 3) MIF solution, and 4) sediment.

d. With the tube in a horizontal position, loosen the plug with an applicator and pour off everything except the sediment. Use a cotton swab to remove debris adhering to the glass.

e. With a capillary pipette, remove the top portion of sediment to a slide, cover with a coverslip, seal and examine.

CONCENTRATION OF PAF-FIXED MATERIAL—PAF-fixed material may be concentrated by simple centrifugal sedimentation (Burrows, 1967a) or by saline-ether sedimentation.

Centrifugal Sedimentation

1. Strain about 10 to 12 ml of PAF-fecal suspension through wet gauze or cheesecloth into a 15 ml centrifuge tube.
2. Add 0.85% saline to bring the fluid volume up to 12 ml.
3. Centrifuge at 1,800 r.p.m. for 3 minutes.
4. Decant the supernatant and check the level of sediment. If less than 0.5 ml, strain another portion of the original suspension into the tube, centrifuge again, and decant the supernatant. The optimum amount of sediment is 0.5 to 0.6 ml.
5. Add saline to the 12 ml level and resuspend the sediment.
6. Centrifuge as above and decant the supernatant.
7. Repeat the washing until the supernatant is clear—2 to 4 times.
8. Add 2 ml of saline to the sediment and mix well.
9. Place a drop of thionin stain (or azure A stain) on a 3×2-inch slide.
10. Using a capillary pipette, transfer a portion of well-mixed sediment to the drop of stain. Mix thoroughly.
11. Cover with a 22 mm-square, #1 thickness coverslip, seal the preparation, and examine in the usual manner.

Saline-ether Sedimentation

1. Strain about 10 to 12 ml of PAF-fecal suspension through wet gauze or cheesecloth into a 15 ml centrifuge tube.
2. Follow the procedure for centrifugation and washing given for the centrifugal sedimentation, adjusting the amount of sediment if necessary (Steps 2 to 7).
3. To the washed sediment, add saline to the 10 ml level on the tube and add 1 drop of Triton NE (or 1 drop of a 33% aqueous dilution of Triton X-100).

4. Mix well to suspend the sediment and let stand for 10 minutes.
5. Add 2 ml of ether, stopper the tube, and shake vigorously for about 30 seconds, keeping the thumb on the stopper.
6. Remove the stopper and centrifuge the tube at 2,000 r.p.m. for 3 minutes.
7. Four layers will result: 1) ether, 2) plug of debris, 3) saline, and 4) sediment.
8. Ring the plug of debris and decant the top 3 layers. Using a cotton swab, wipe the inside of the tube almost to the sediment layer.
9. Add 1 or more drops of saline to the sediment, if necessary, and mix the sediment and fluid thoroughly.
10. With a capillary pipette, transfer a portion of the sediment to a slide. Cover slip and seal the mount.
11. A drop of thionin stain can be added to the preparation to stain protozoa.

STAINING PROCEDURES

FECES

Permanently stained preparations facilitate detection and identification of cysts and trophozoites and afford a permanent record of the organism encountered. Small organisms, missed by other examinations, may be found on stained films. The classical staining technique is the long Heidenhain's iron-hematoxylin method, but, for routine diagnosis, shorter procedures such as the trichrome method (Wheatley, 1951), the phosphotungstic acid technique (Tompkins and Miller, 1947), and the chlorazol black stain technique (Kohn, 1960; Gleason and Healy, 1965) have been developed. The solutions and schedules for these methods are presented below.

PREPARATION OF SMEARS—For permanent stain preparations, use 3×1-inch slides. They should be clean and grease free. Frosted end slides are recommended, since the identifying information can be written on the frosted area with an ordinary lead pencil. If non-frosted end slides are used, etch the identification into the glass with a diamond-point pencil. China- or glass-marking pencil marks will wash off during staining.

Fresh Specimens—Using an applicator stick, make a thin, even smear of the fecal sample by streaking the material back and forth on the slide. If necessary, dilute feces with physiological saline (0.85%). A thin, even smear of feces is essential for good preparations. An absolutely uniform density cannot be obtained, but the feces should be as evenly distributed on the slide as possible. Smears that are too thick stain poorly and will be too dense to examine adequately. Thin smears destain too rapidly, and, unless organisms are numerous, they may be missed or require prolonged searching to be found. Immerse slide into Schaudinn's fixative (Solution #1) *immediately* after preparation. *The smear must not be permitted to dry from the time it is made until it is mounted. This is very important.* Slides may be left in the fixative for several hours or days, if necessary. If the staining schedule for an iron-hematoxylin procedure must be interrupted before or after the mordanting-staining process, slides may be stored for long periods in 70% alcohol without subsequently changing the regular schedule. If the schedule must be interrupted between the mordant and stain, store slides in 70% alcohol but repeat the mordanting step when ready to stain. In the trichrome procedure, the slides may be left in the second 70% alcohol (step 4).

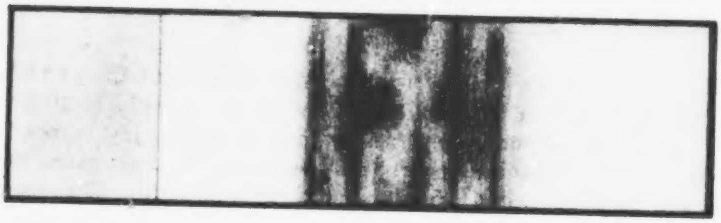
Arensburger and Markell (1960) have developed a procedure for concentrating cysts, mixing the concentrate with fresh feces, and making stained preparations of this mixture. The procedure is as follows:

1. Mix about 1 ml or 1 gm of feces (a portion about the size of a marble) in 10 to 15 ml of tap water.
2. Stain approximately 10 ml of the suspension through 2 layers of wet gauze (1 layer of fine-mesh, closely woven gauze is sufficient) into a 15 ml centrifuge tube.
3. Add 1 to 2 ml of ether. Stopper and shake thoroughly.
4. If necessary, add water to fill the tube to a level approximately 1 cm below the top.
5. Centrifuge at 2,500 r.p.m. for 45 seconds.
6. Loosen the plug, which forms at the top, and decant the supernatant fluid.
7. Add 2 to 3 ml of physiological saline to the sediment and mix thoroughly.
8. Fill the tube to 1 cm from the top with saline.
9. Centrifuge at 2,500 r.p.m. for 45 seconds and decant the supernatant.
10. With an applicator stick, add a small quantity of the original fecal specimen to the sediment in the tube. Mix thoroughly to form a homogeneous suspension of feces and sediment.
11. Transfer the mixture, or a portion of it, to a 3×1-inch slide and prepare a smear as described above for fresh specimens.
12. Place the smear immediately in Schaudinn's fixative.

Smears prepared from this material may be stained by the usual staining procedures. The use of water to prepare the initial suspension eliminates *Blastocystis* and some of the debris. The saline, used in the successive steps, decreases the osmotic effect that might damage trophozoites, if present in the original feces, when the concentrate and feces are mixed.

PVA-fixed Specimens—With an applicator stick, spread (not smear) 2 or 3 drops of the preserved specimen over approximately one-third of the surface of a microscope slide, taking care to extend the smear to the edges of the slide (Figure 10). The films are allowed to dry thoroughly for several hours or overnight either in a 37°C incubator or at room temperature. The films should *dry thoroughly* to prevent the material from washing off the slide during staining. Slide may be left unstained up to 2 months.

Figure 10.
Properly Prepared Smear of PVA-Fixed Feces



A more rapid procedure for preparing PVA-fixed fecal smears has been developed by the California State Health Department Laboratory (1965). Thoroughly shake the bottle of PVA-fixed specimen and pour 2 to 4 ml into a small tube (85×15 mm). Centrifuge for 5 minutes at 2,400 r.p.m. and decant the PVA fluid. Remove the PVA solution adhering to the glass by swabbing the inside of the tube with a cotton swab. Using an applicator stick, remove a portion of the sediment to a piece of facial tissue to absorb excess PVA. With an applicator stick, smear the partially dry fecal material on a coverslip (or slide) in the manner described for fresh fecal specimens. If the specimen is mucoid, fatty, or watery, coating the coverslip with a *very thin* film of clear human serum before making the smear will cause the fecal material to adhere better. The serum is applied by touching a cotton swab dipped in serum to the coverslip (or slide) and wiping off the excess. Too much serum will produce a poor stain. The preparation can be stained immediately without drying. The procedure for fresh fecal smears can be used rather than the longer schedule usually recommended for PVA smears.

Slides of PVA-fixed material may be stored in 70% alcohol as described for fresh specimens, if the staining schedule must be interrupted.

EXAMINATION—Permanently stained smears should be examined with the oil immersion objective. Although cysts and trophozoites of *E. coli* and trophozoites (and occasionally cysts) of *E. histolytica* can be detected under low or high dry magnification, structural details cannot be seen clearly except with oil immersion magnification. Also, organisms may be overlooked in stained smears if low power is used to locate them, so for practical purposes, stained preparations need to be examined only with oil immersion objectives. The use of a 43X-, 44X-, or 50X-oil immersion objective will speed up the examination, however. Some people find that a 5X- or 6X-ocular in combination with the 97X- or 98X-oil immersion objective facilitates examination.

In examining the smears, light and dark (thin and thick) areas should be looked at. Usually organisms are more easily located in thin areas, but in some preparations these portions may be poorly stained or the organisms poorly differentiated. In these cases, the thicker portions would be of more diagnostic value. Organisms are not uniformly mixed with feces, so the examiner should examine several areas of the mount; for example, top, center, and bottom or left, center, and right areas. For a reliable report, a competent microscopist should examine the smear for at least 15 to 20 minutes, especially if organisms are not found.

PREPARATION OF SOLUTIONS

1. Fixative (Schaudinn's Solution)

Ethyl alcohol, 95%	1 part
Saturated aqueous mercuric chloride (See page 60 for preparation.)	2 parts

Before use, add 5 ml of glacial acetic acid per 100 ml of solution.

2. Iodine Alcohol

Prepare a stock solution by adding enough iodine crystals to 70% alcohol to make a dark, concentrated solution. For use, dilute some of the stock with 70% alcohol until a strong tea-colored solution is obtained. The exact concentration is not important, but the solution should not be too dark, since the iodine may stain the protozoa and interfere with subsequent hematoxylin or trichrome staining.

3. Mordant

Ferric ammonium sulfate (violet crystals)	4.0 gms
Distilled water	100.0 ml

Dissolve the ferric ammonium sulfate crystals in the distilled water.

This solution should be prepared fresh just before use.

Note: Ferric ammonium sulfate crystals should be stored in the refrigerator.

4. Stains

A. Stock Hematoxylin

Hematoxylin (powder or crystals)	10 gms
95% or 100% ethyl alcohol to make	100 ml

Dissolve hematoxylin in a small quantity of alcohol. Add alcohol to make 100 ml. Let ripen for 6 weeks before diluting for use in staining. When ripe, the stain is a deep orange-brown, clear solution.

B. Hematoxylin Staining Solution (0.5%)

Stock solution, ripened	5.0 ml
Distilled water	95.0 ml

Add stock hematoxylin to the distilled water and mix. This makes a 0.5% staining solution. The dilute aqueous solution is not stable and must be prepared fresh each day or when needed.

C. Trichrome Stain

Chromotrope 2R	0.6 gm
Light Green SF	0.15 gm
Fast Green FCF	0.15 gm
Phosphotungstic acid	0.7 gm
Acetic acid (glacial)	1.0 ml
Distilled water	100.0 ml

Put the dry stains into a clean flask. Add the glacial acetic acid, shake to mix, and allow the mixture to stand ("ripen") for 30 minutes. Add the distilled water. Shake to mix thoroughly. The stain is stable and is used without diluting. Good stain is deep purple, almost black.

D. Chlorazol Black E Stain

(1) Basic Solution

90% ethyl alcohol	170 ml
Methyl alcohol	160 ml
Glacial acetic acid	20 ml
Liquid phenol	20 ml
1% phosphotungstic acid*	12 ml
Distilled water	618 ml

Add alcohols and acids to distilled water and mix thoroughly.

(2) Stock Stain Solution

Chlorazol black E dye	5 gms
Basic solution	1,000 ml

Weigh out dye, put in mortar, and grind for at least 3 minutes. Add a small amount of basic solution and grind until a smooth paste is obtained. Add more solution and grind for 5 minutes. Allow particulate matter to settle a few minutes and pour off the liquid into a separate dry, clean container. Add more basic solution and continue the grinding, mixing process until all of the dye appears to be in solution. Add any remaining basic solution, bottle the stain, and put aside for 4 to 6 weeks to ripen. A black sediment settles out within a few days, leaving a black cherry-colored liquid which is the fixative-stain. Filter the stain through Whatman #12 filter paper before use. Keep the filtered stain in a stoppered bottle protected from moisture, dust, and dirt.

5. Destaining Solutions

A. Ferric Alum Solution (2% Solution)

Dilute mordant (solution #3) 1:1 with distilled water.

B. Phosphotungstic Acid Solution

Phosphotungstic acid	2.0 gms
Distilled water	100.0 ml

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*1 gm phosphotungstic acid crystals dissolved in 100 ml distilled water.

C. Saturated Aqueous Picric Acid Solution

Add approximately 2 gms of picric acid crystals to 100 ml of water. Shake thoroughly; let stand for several days, shaking at intervals. If all the crystals dissolve, add more. After 3 to 4 days, some undissolved picric acid should remain in the flask. Let settle. Use the clean solution from top of the bottle, or filter.

D. Acid Alcohol

Acetic acid	0.45 ml
90% ethyl alcohol	99.55 ml

6. Carbol-Xylene

Add 1 volume of liquefied carbolic acid (phenol) to 3 volumes of xylene. Phenol crystals can be liquefied by heating the jar in a water bath. *Loosen the jar lid!*

For chlorazol black stain, 1:2 carbol-xylene is preferred.

Note: Liquid phenol solutions which contain water *cannot* be used in carbol-xylene solutions.

7. Lithium Carbonate, Saturated Aqueous Solution

Dissolve lithium carbonate powder in distilled water until no more will go into solution. About 1 to 2 gms per 100 ml water is usually sufficient.

TECHNIQUES

A. Heidenhain's Iron-Hematoxylin Stain

The Heidenhain's iron-hematoxylin technique, a standard, long-used procedure, is described in many textbooks. The "classical" long method gives excellent staining results if the procedure is carefully followed, especially with regard to the destaining of the organisms. By heating the fixative, mordant, and stain, the procedure can be shortened considerably, however, without loss of staining details. Both the long and short Heidenhain's techniques for fresh fecal specimens are given below. PVA-fixed material can be stained by either method, but better results will be obtained by using the procedure particularly adapted for PVA specimens. (This procedure is given separately.)

Technique

1.	Schaudinn's fixative (Solution #1)	5 minutes at 50°C, 1 hour at room temperature
2.	70% alcohol plus iodine (Solution #2)	5 minutes
3.	50% alcohol	3 minutes
4.	Tap water	3 minutes
5.	4% iron alum mordant (Solution #3)	10-20 minutes at 40-50°C, 12-24 hours at room temperature
6.	Water (distilled or tap) two changes	3 minutes (total)
7.	0.5% hematoxylin (Solution #4b)	5-10 minutes at 40-50°C, 12-24 hours at room temperature
8.	Tap water, two changes	3 minutes (total)
9.	1%-2% iron alum destain (Solution #5a)	By observation under the microscope (usually requires 1-3 minutes or more)
	—or—	
	Saturated aqueous picric acid (Solution #5c)	5-10 minutes or more by observation under the microscope

This is the critical step in this technique and the process must be checked carefully. At intervals of ½ minute, 1 minute, 2 or more minutes, depending on the destain solution used, the smear should be removed from the solution, rinsed thoroughly with water to slow down or stop the destaining process, and examined microscopically with low or high power. Repeat the process until optimum results are obtained. The destaining is completed when the structural details in the organism, such as nuclei, are visible, or, if organisms cannot be located, until the background material is more gray than black. The time will vary widely depending on the smear, the organisms, and other factors. *Experience and practice are necessary to get the best results.* An experienced technician may destain visibly without microscopic observation. If time permits, several films may be prepared from one specimen and destained by varying the time, that is, for 1 minute, 2 minutes, etc. Usually at least one slide from a group is properly destained. This is time consuming, however, and the destain process should be controlled by microscopic observation.

10. Running tap water (or several changes) .... At least 5-30 minutes (for permanence of stain)
11. 70% alcohol plus a few drops of saturated lithium carbonate solution (#7) per stain jar .... 3 minutes
12. 95% alcohol .... 5 minutes
13. Carbol-xylene .... 5 minutes
14. Xylene .... 3 minutes
15. Mount with coverslip, using Permount or other suitable mounting media.

Stain Reactions (Iron-Hematoxylin)

Well-stained organisms are bluish or grayish with black nuclear structures. Chromatoid bodies of amebae cysts and inclusions, such as bacteria or red blood cells, in the cytoplasm of trophozoites stain black. Background material usually stains blue-gray.

Protozoan cysts and trophozoites are generally not distorted with the exception of trophozoites of *Chilomastix* and *Trichomonas*. The latter tend to round up and are often atypical in appearance. Non-staining organisms may indicate poor fixation. With fresh stools, poor fixation can be largely overcome by heating the fixative to 56°C and fixing for 5 to 10 minutes. Cold fixation (room temperature) with fresh fixative, however, gives more critically stained organisms. With the exception of *E. coli*, 30 minutes' cold fixation is usually sufficient. For *E. coli*, 2 to 3 hours at room temperature or ½ hour at 56°C may be necessary to insure thorough fixation of cysts. Immature and young cysts fix more easily than old cysts, and trophozoites frequently are fixed in 2 to 3 minutes.

Mononuclear and polymorphonuclear leucocytes, *Blastocystis*, yeasts, and molds may cause confusion in diagnosis unless the observer is trained in differential morphology of the protozoa.

B. Modified Heidenhian Stain for PVA-fixed Material

The staining of dried PVA films is essentially the same as for other types of fecal smears except for a variation in the time required for certain steps and the omission of the usual Step 1, or fixation in Schaudinn's solution. Use of PVA-fixative solution as a preservative makes further fixation unnecessary.

Technique

1.	70% alcohol plus iodine (Solution #2)	20 minutes
2.	50% alcohol	10 minutes
3.	Tap water	5 minutes
4.	4% iron alum mordant (Solution #3)	8-12 hours
5.	Water (distilled or tap), two changes	3 minutes (total)
6.	0.5% hematoxylin (Solution #4b)	8-12 hours (or overnight)
7.	Tap water, two changes	3 minutes (total)
8.	Saturated picric acid destain (Solution #5c)	15-20 minutes, or by observation under the microscope

This is the critical step in this technique. See the note with step 9 of the standard procedure. The time may vary depending upon the smear, the organism, and other factors. Experience and practice are necessary to get the best results.

9.	Running tap water, or several changes	30 minutes (for permanence of stain)
10.	70% alcohol plus a few drops of saturated lithium carbonate solution per stain jar	10 minutes
11.	95% alcohol	10 minutes
12.	Carbol-xylene	10 minutes
13.	Xylene	10 minutes
14.	Mount in Permount or other mounting media.	

Stain Reactions

The organisms stain as described above. However, cysts such as *E. histolytica* and *E. coli* may be distorted in PVA preparations.

C. Iron-Hematoxylin-Phosphotungstic Acid

The phosphotungstic acid technique of Tompkins and Miller (1947) is a good short hematoxylin staining procedure for routine diagnostic purposes. The destaining is self-limiting and does not require control by microscopic observation as does the classical technique. The stain reaction is similar to that described by Heidenhain's stain except that the slides are bluer in appearance. This procedure gives better results with fresh fecal smears than with preparations from PVA-fixed feces.

Technique

1.	Schaudinn's fixative (Solution #1)	5 minutes at 50°C, 1 hour at room temperature
2.	70% alcohol plus iodine (Solution #2)	5 minutes
3.	50% alcohol	3 minutes
4.	Tap water	3 minutes
5.	4% iron alum mordant (Solution #3)	3-5 minutes at room temperature
6.	Distilled or tap water	1 minute
7.	0.5% hematoxylin (Solution #4b)	1 minute at room temperature
8.	Distilled or tap water	1 minute
9.	2% phosphotungstic acid destain (Solution #5b)	2 minutes or more
10.	Running tap water (or several changes)	5 minutes (total)
11.	70% alcohol plus a few drops of saturated lithium carbonate solution per stain jar	3 minutes
12.	95% alcohol	3 minutes
13.	Carbol-xylene	5 minutes
14.	Xylene	3 minutes
15.	Mount with coverslip using Permount or other suitable mounting media.	

Stain Reactions

The organisms stain as described above but the preparations are bluer than those obtained by the longer methods.

D. Trichrome

The trichrome technique of Wheatley (1951) is a rapid staining procedure which gives good results for routine purposes. The method is simple in that overstaining and differentiation are not necessary to bring out the morphologic details of the parasites, nor is it necessary to mordant before staining. However, for practical purposes, destaining the smears gives better differentiation of the organisms, and this step should be included. The stain solution is stable and may be used repeatedly, the lost volume being replaced by adding stock solution. Staining over 15 smears daily (in 50 ml of stain), however, tends to weaken the stain. If stain is allowed to evaporate in the open air for 3 to 8 hours, strength will return. This can be accomplished by leaving the cover of the staining dish off overnight. The staining of fresh and PVA-fixed material differs chiefly in the increased time required for the latter and in the omission of the fixative step, since the material in the PVA solution is already fixed. Both procedures are given below.

Staining Technique with Fresh Specimens

1. Schaudinn's fixative (Solution #1)	5 minutes at 50°C, 1 hour at room temperature
2. 70% alcohol plus iodine (Solution #2)	1 minute
3. 70% alcohol (1)	1 minute
4. 70% alcohol (2)	1 minute
5. Stain (trichrome) (Solution #4c)	2-8 minutes
6. 90% alcohol, acidified (Solution #5d)	10-20 seconds or until stain barely runs from smear

Prolonged destaining in 90% alcohol (over 20 seconds) may cause the organisms to be poorly differentiated, although larger trophozoites, particularly those of *E. coli*, may require slightly longer periods of decolorization. Since the acid alcohol continues to destain as long as it is in contact with the material, the time allowed should include the few seconds between the time the slide is removed from the destain and the rinse in 95% alcohol (step 7). If several slides are being stained, the 95% alcohol (step 7) may have to be changed to prevent it from becoming so acid that the destaining process will continue.

7. 95% or 100% alcohol	Rinse twice
8. 100% alcohol or carbol-xylene	1 minute
9. Xylene	1 minute or until refraction at smear-xylene interface ends.
10. Mount with coverslip using Permount, or other mounting media.	

Staining Techniques with PVA Films

1. 70% alcohol plus iodine (Solution #2)	10 minutes
2. 70% alcohol (1)	3-5 minutes
3. 70% alcohol (2)	3-5 minutes
4. Trichrome stain (Solution #4c)	6-8 minutes
5. 90% alcohol, acidified (Solution #5d)	10-20 seconds or until stain barely runs from smear
6. 95% alcohol (1)	Rinse to remove acid destain
7. 95% alcohol (2)	5 minutes
8. Carbol-xylene	5-10 minutes
9. Xylene	10 minutes
10. Mount with coverslip using Permount or other mounting media.	

Stain Reactions (Trichrome)

The cytoplasm of thoroughly-fixed and well-stained cysts and trophozoites is blue-green, tinged with purple. Occasionally, *E. coli* cysts may stain slightly more purplish than cysts of other species. The nuclear chromatin, chromatoid bodies, and ingested red cells and bacteria stain red or purplish red. Other ingested particles, such as yeasts or molds, generally stain green, but variations frequently occur in the color reaction of ingested particles. Background material usually stains green, thus contrasting with the protozoa.

Non-staining cysts and those staining predominately red are most frequently associated with incomplete fixation. Unsatisfactorily stained organisms obtained from specimens submitted in PVA-fixative usually indicate incomplete fixation associated with poor emulsification. Thorough emulsification of preferably *soft* stools will yield critically stained cysts and trophozoites. Degenerate forms stain pale green, although understained or overstained organisms may also appear green.

Eggs and larvae stain red and contrast strongly with the green background. Thin-shelled eggs often collapse when placed in mounting medium, although some diagnostic features may be retained, especially if the smear is examined immediately.

In contrast to organisms stained with hematoxylin, trichrome smears have a transparency which enables imbedded protozoa to be identified even in thicker smears. Protozoa and eggs are less subject to distortion, however, in thinner smears.

Mononuclear and polymorphonuclear leucocytes and *Blastocystis* present the same diagnostic problems that they present when stained with hematoxylin. The cytoplasm of pus and tissue cells, however, does stain more greenish than that of the protozoa.

E. Chlorazol Black E Stain

Chlorazol black E stain technique (Kohn, 1960) is a simple procedure in which fixation and staining take place in a single solution. No destaining is necessary. The modified stain technique (Gleason and Healy, 1965) may be used for staining protozoa in fecal smears or in tissues. The stain gives good results with fresh fecal smears but is less satisfactory for PVA-fixed material. (PVA-fixed specimens are better stained by iron-hematoxylin or trichrome stains.)

The optimum staining dilution and time for each liter, or "batch", of stain must be determined. The stock stain may be kept indefinitely, and stain dilutions may be used repeatedly. However, repeated use "wears out" the stain dilution, and when slides appear visibly red rather than greenish-black at the end of the staining period, the dilution should be discarded. Approximately 20 slides can be stained satisfactorily in a 50-ml coplin jar of diluted stain before deterioration makes change necessary. Deterioration of the stain is dependent on use (slides stained) and not on time, since satisfactory stains may be obtained with stain dilutions up to 30 days old if the number of slides stained has not been excessive. Slides that appear red can be restained in fresh stain.

1. Technique for Staining Fecal Smears

a. Determination of Optimum Dilution and Staining Time

The optimum dilution and staining time must be determined for each liter of fixative-stain. The following series of dilutions and staining periods are recommended for this purpose.

Fixative-Stain [Solution 4d(2)]	*Basic Solution* [Solution 4d(1)]	*Hours*
Undiluted	—	2-3
1	1	2-4 to overnight
2	1	2-4
1	2	2 to overnight
1	3	4 to overnight

A solution producing good overnight staining does not appear to overstain when left for several days.

Trial smears should be stained with each dilution according to the technique given below and the optimum dilution and time selected for routine staining with the "batch" of stain concerned. More than one dilution-time combination may be satisfactory and the choice of which to use will depend on the laboratory schedule and urgency of diagnosis. The range most commonly found satisfactory is a 1:2 dilution for 2 hours or a 1:3 for 4 hours or overnight. However, for best results, the exact combination should be determined.

b. Techniques of Staining

1) Fixative-stain dilution (for routine use, use dilution and time predetermined) .... 2 hours to overnight
2) 95% ethyl alcohol .... 10-15 seconds
3) Carbol-xylene (1:2) .... 5 minutes
   —or—
   100% ethyl alcohol .... 5 minutes
4) Xylene .... 5 minutes
5) Mount in Permount or other suitable media.

2. Technique for Staining of Protozoa in Tissue

Tissue slides are prepared following the usual histological procedures for fixation, embedding, sectioning, and preparation of slides. The sections are treated as usual in xylene and alcohols to remove the paraffin and prepare them for staining. Good stains have been obtained on sections of 5 to 7 microns in thickness.

a. Determination of Optimum Dilution and Staining Time

For staining protozoa in tissue, use the optimum stain dilution as determined for protozoa in feces, but substitute distilled water for the basic solution as the diluent.

Fixative-Stain	*Distilled Water*	*Hours*
Undiluted	—	4-6
1	1	4-8 to overnight
2	1	4-8
1	2	4 to overnight
1	3	8 to overnight

Note that the staining time is twice as long for tissues as for fecal smears. The most common satisfactory time is about 6 to 7 hours with the optimum dilution.

b. Technique of Staining

1) Fixative-stain	4 hours to overnight (as determined)
2) 95% alcohol	10-15 seconds
3) Carbol-xylene (1:2)	5-10 minutes
4) Xylene	5-10 minutes

5) Mount in Permount or other suitable media.

Stain Reactions

Protozoa in fresh fecal specimens stain green to gray-green; in older stools, organisms are gray to black. Nuclei, chromatoid bodies, karyosomes, and cell membranes stain dark green to black. Ingested red cells may vary from pink to black. *E. coli* cysts may stain pink or green, and rarely, *E. histolytica* cysts stain faintly pink. *E. histolytica* trophozoites have the same appearance in tissue as in feces.

ADULT HELMINTHS

Preparing stained helminths involves a procedure similar to that used for fecal smears. Prior to staining, however, the worms must be relaxed and carefully fixed. Some of the more common methods of relaxation, fixation, and staining are described below. To secure good preparations, start with clean organisms. Using a soft brush of fine needles, carefully remove the mucus and adherent debris from the worms in saline.

PREPARATION OF SOLUTIONS

1. Fixatives

a. Formalin—5 to 10%

b. Alcohol-Formalin-Acetic Acid (AFA)

Commercial formalin	10 parts
95% ethyl alcohol	25 parts
Acetic acid (glacial)	5 parts
Glycerin	10 parts
Distilled water	50 parts

2. Stains

a. Alum Cochineal

Potassium alum	30 gms
Cochineal	30 gms
Distilled water	400 ml

Boil for 1 hour. Cool and filter. Boil the filter paper in 200 ml distilled water for 30 minutes. Filter. Add the second filtrate to first filtrate and boil 30 minutes. Filter and make up to 400 ml with distilled water.

b. Delafield's Hematoxylin

1) Prepare a saturated aqueous solution of ammonium alum as follows: Add aluminum ammonium sulfate (ammonia alum) crystals to hot distilled water, stirring continuously, until no more crystals will go into solution. Let cool. Excess alum will crystallize out. Pour off the clear solution or filter. Store in a glass stoppered bottle. The solution will keep indefinitely.

2) Stain

Hematoxylin crystals	4 gms
95% ethyl alcohol	25 ml
Ammonium alum, saturated aqueous solution	400 ml

Dissolve the stain crystals in the alcohol and add the ammonium alum solution. Place in loosely capped container and keep in sunlight for 2 weeks. Then add a mixture of:

Methyl alcohol (acetone free)	100 ml
Glycerin	100 ml

Place in a glass stoppered bottle and expose to direct sunlight for at least a month. Filter before use.

c. Reynold's Stain

Alum cochineal	3 parts
Delafields's hematoxylin	1 part
Distilled water	25 parts

This mixture may be used immediately, but it is best to allow it to stand for a few hours after mixing. A fine precipitate will settle out but it can be removed by filtering. The mixture is then ready for use.

d. Semichon's Acetic Carmine

Acetic acid (glacial)	1 part
Distilled water	1 part

Place in small flask; add excess carmine powder. Stopper, and place flask in boiling water for 15 minutes. Cool, decant supernatant, and filter.

TECHNIQUE

1. Relax and extend organisms by *one* of the following methods:
    a. Small helminths may be placed in a drop of saline on a slide, a coverslip put on top so that *gentle* pressure is exerted, and fixative run under the coverslip.
    b. Organisms may be placed in a bottle half-filled with saline and shaken vigorously for 2 to 3 minutes.
    c. Worms or proglottids may be placed in water and put in the refrigerator for 30 minutes to overnight.
    d. Tapeworms may be wrapped about a glass jar or slide. Strips of proglottids may be gently flattened and extended between paper towels soaked in fixative. Such strips may be placed between large pieces of glass or slides which are then tied lightly but firmly together with string to flatten the organisms slightly. Keep moist with fixative solution.
2. Fix for the time necessary for the fixative used. Transfer the worms or segments to the fixative after relaxation. If the organism was relaxed under a coverslip or between slides, run the fixative under the coverslip or slide.
    a. **Formalin.** The solution should be steaming, but not hot. With the worms in extended position, pour the solution over them. After several hours, pour off the fixative and add fresh, unheated formalin, preferably 5%. If the container is sealed to prevent evaporation, the organisms can be kept indefinitely.

b. **Alcohol-Formalin-Acetic-Acid.** Use cold. Organisms may remain in the fixative for several days without injury; ordinarily 12 to 24 hours is sufficient. If parasites are to be stored, replace the AFA with 70% alcohol.

3. 50% alcohol .... 1 hour
4. Distilled water .... 10 minutes
5. Stains. One of the following stains may be used; the time required for staining depends on the stain used and the thickness of the organism.

   a. **Alum-cochineal.** Staining usually requires 24 to 36 hours depending on the size and density of the parasite. After staining, wash the organism in water for 15 to 20 minutes, then place it in 70% ethyl alcohol. With this stain, destaining (Step 7) is usually not necessary and may be omitted.

   b. **Delafield's hematoxylin.** Stain for 1 hour or longer, depending on the size of the organism. Organisms may be left in diluted stain for much longer periods and watched until staining has reached the desired point.

   c. **Reynold's Stain.** Stain 4 hours to overnight. Small objects may stain in 4 hours, but most organisms should stain overnight.

   d. **Semichon's Acetic Carmine.** Stain 24 hours.

6. 70% ethyl alcohol .... 2 minutes
7. Acid alcohol (1 ml concentrated HCl to 99 ml of 70% ethyl alcohol) ...... until internal structures are visible by strong transmitted light
8. 70% alcohol .... 1-2 minutes
9. Neutralize in 70% alcohol to which lithium carbonate has been added. (Add several drops of a saturated aqueous solution of lithium carbonate to each 100 ml alcohol. See page 107 for preparation.) .... 1 hour
10. 95% alcohol .... 1 hour
11. 100% alcohol .... 30 minutes
12. 100% alcohol .... 1 hour
13. 100% alcohol plus cedarwood oil (for clearing), equal parts .... ½-1 hour
14. Cedarwood oil .... 1 hour
15. Mount in Permount, Balsam, or other suitable media.

    NOTE: Do not allow the organism to dry at any time until it has been mounted.

CLEARING HELMINTHS WITHOUT STAINING

Clearing helminths without staining reveals most structural details, and the species can ordinarily be identified more quickly by clearing than by using lengthy staining procedures. The cleared specimen should be rotated and oriented in the preparation for optimum observation of details.

Tapeworm proglottids and small worms may be cleared in the following solutions. Clearing can be observed visually.

Carbol-xylene (1:3): Clearing is very rapid. The uterine branches of proglottids can be seen easily by holding the segment between 2 slides towards the light.

Lacto-phenol (1:1): An equal volume of glycerin may be added. This solution is good for nematodes, but prolonged contact may clear the organism too much.

Beechwood cresote: Clearing is slower than with the above solutions, but organisms remain soft and pliable.

Xylene: Clearing occurs fairly quickly, but the specimen may become brittle. Tapeworm proglottids, however, are cleared effectively with xylene.

Glycerin: Fairly satisfactory with nematodes, such as hookworm, but may occasionally cause shrivelling or wrinkling.

PVA-lacto-phenol: This is a fairly good clearing and mounting medium for small nematodes. The relative proportions of PVA* and lacto-phenol may be varied to give the degree of clearing desired.

Rubin (1951) advocated the following proportions:

PVA, 15% aqueous solution	56 parts
Lactic acid	22 parts
Phenol	22 parts

In the NCDC laboratory, less clearing and better detail was obtained with a medium of the following proportions:

PVA, 15% aqueous solution	70 parts
Lacto-phenol (1:1)	30 parts

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*PVA powder of the same grades as recommended for use in PVA-fixation solution is used.

CULTIVATION PROCEDURES

PROTOZOA

Various media have been used in cultivating amebae and intestinal or atrial flagellates. The coccidia, *B. coli*, and *G. lamblia* either cannot be cultured or require special media. Some of the commonly used media for amebae and flagellates are included here.

MODIFIED BOECK AND DRBOHLAV'S MEDIUM—This medium consists of a solid egg base and an overlay of Locke's solution (Boeck and Drbohlav, 1925; Reardon and Rees, 1939). It supports growth of most of the intestinal protozoa and satisfactorily maintains them. Since it does not require fresh serum and uses only materials usually available in laboratories, it is recommended for routine purposes.

Glassware and material used in the preparation should be sterile because the length of time the medium is sterilized may not be adequate to kill large numbers of bacteria in the solid egg base. Experience with the efficiency of one's autoclave will determine whether aseptic precautions are necessary.

Modified Locke's Solution (Overlay)

Sodium chloride	8.0	gms
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.2	gm
Potassium chloride	0.2	gm
Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	0.01	gm
Sodium phosphate (Na_2HPO_4)	2.0	gm
Sodium bicarbonate	0.4	gm
Potassium phosphate (KH_2PO_4)	0.3	gm
Distilled water	1,000.0	ml

Add the above chemicals in the order listed to the distilled water. Mix each until dissolved. Boil 10 minutes. A precipitate will form. Cool to room temperature and filter through paper. Sterilize in an autoclave for 15 minutes at 15 lbs (121°C).

Preparation of Egg Slants

1. Wash 4 fresh eggs and carefully break them in a 1,000 ml flask containing a few glass beads or into a Waring Blendor. It is usually easier to break 1 egg at a time into a beaker and then transfer it to the flask through a small funnel. Any eggs that do not appear fresh should be discarded. A "blood spot" in a fresh egg does not harm the medium.
2. Add 50 ml of sterile Locke's solution and emulsify by shaking the flask.
3. Filter the mixture through gauze into a dispenser.
4. Dispense into sterile test tubes (15×125 mm) in such a quantity that there will be a short butt and a 4 to 5 cm slant.
5. Inspissate. If an inspissator is not available, inspissate in the autoclave as follows:

   If the autoclave has a jacket, allow the pressure to reach 15 lbs. Place the tubes in the chamber in the desired slanted position. Close all exhaust valves and the door to the chamber. This procedure will entrap the air in the chamber. Allow the steam from the jacket to enter the chamber as fast as possible until the pressure gauge registers 15 lbs. Hold at this pressure for 15 minutes. Cut off the steam supply and with all doors and valves still tightly closed, allow the pressure to drop gradually.

   If the autoclave has no jacket, place the tubes in the chamber and close all exhaust valves and the door tightly. If the drain at the bottom of the autoclave has no valve, stopper it with a rubber stopper. Turn on the steam and allow the pressure to rise to 15 lbs. The temperature of the mixture of air and steam will not be above 85°C. Hold at this pressure for 15 minutes. Turn off the steam and allow autoclave to cool gradually. Sudden drops of pressure tend to disrupt the medium causing bubbles and/or rough surfaces.
6. Allow the slants to cool and cover with Locke's solution so that the overlay forms a column 5 to 6 cm deep.
7. Sterilize in the autoclave under 15 lbs of pressure for 15 minutes (temperature 121°C). Allow the pressure to drop gradually.
8. Incubate at 37°C for 24 hours and check for sterility. If the fluid is clear the medium can be stored in the refrigerator for future use. Fresh medium is more satisfactory than stored medium, but, if necessary, it can be kept for a month.

Sterile Rice Starch

Difco Rice starch (or its equivalent) is used. Spread a few grams evenly in a large tube and heat in a hot air oven for 2½ hours at 150°C. Keep the tube in a horizontal position during sterilization. If larger quantities are needed, use the dispenser, funnel type container as shown in Figure 11. Sterilize in the autoclave at 121°C for 20 minutes.

EGG YOLK INFUSION MEDIUM—This is an all liquid, egg yolk infusion medium to which liver has been added (Balamuth, 1946). It is good for isolating most intestinal protozoa, and, furthermore, it may be stored for several months without marked deterioration.

Liver Extract Solution

Wilson's Dry Extract of Liver (powder)	5 gms
Distilled water	100 ml

Dissolve the liver extract powder in the distilled water and heat to the boiling point. Filter through ordinary filter paper and autoclave at 15 lbs. (121°C) for 20 minutes.

Buffer Solutions

1. Stock Buffers (1 M Solutions)

   a. Dibasic phosphate solution

K_2HPO_4	87.09 gm
Distilled water to make	500 ml

In a 500 ml volumetric flask, dissolve the K_2HPO_4 in part of the distilled water. Add water to make 500 ml of solution.

   b. Dihydrogen phosphate solution

KH_2PO_4	13.61 gms
Distilled water to make	500 ml

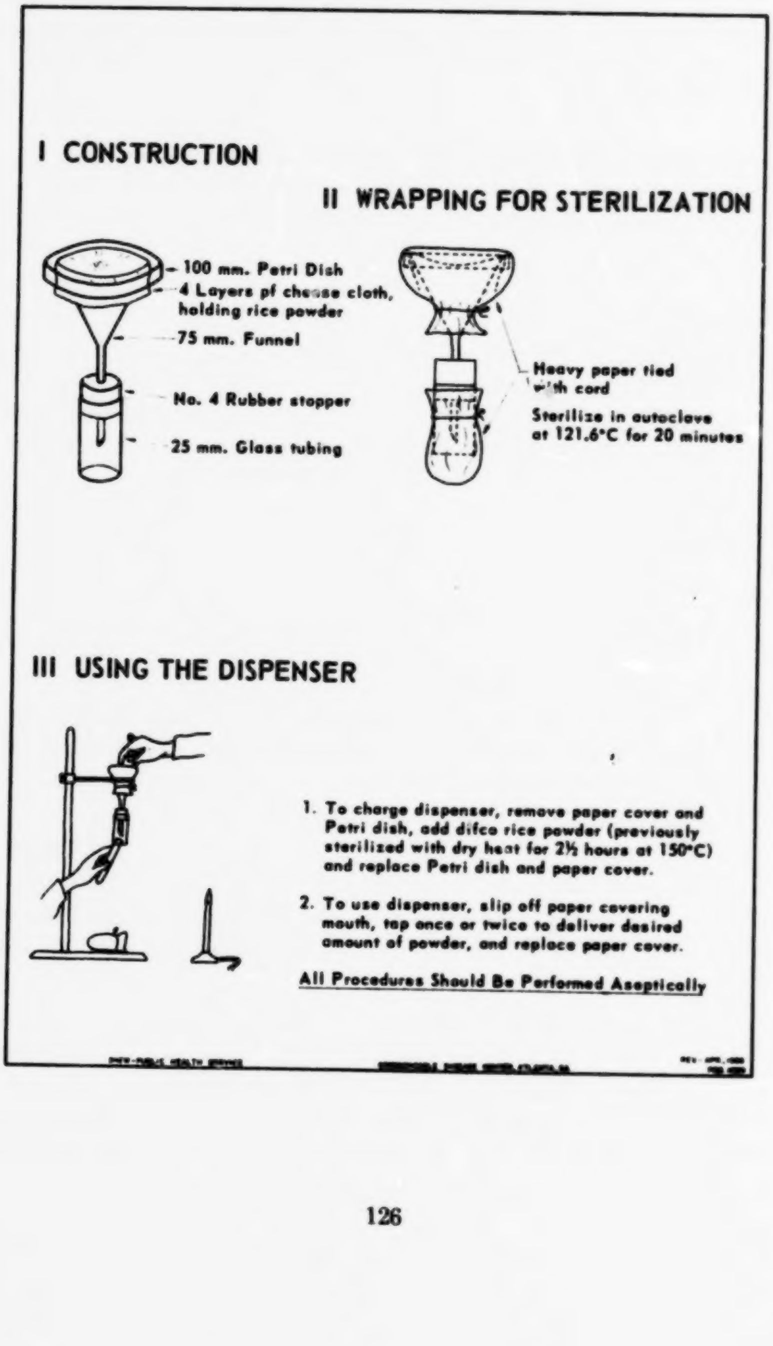
In a 500 ml volumetric flask, dissolve the KH_2PO_4 in part of the distilled water. Add water to make 500 ml of solution.

2. Buffer Solution (M/15 Solution)

Dibasic phosphate (K_2HPO_4) stock buffer	4.3 parts
Dihydrogen phosphate (KH_2PO_4) stock buffer	0.7 part

Mix the stock buffers and dilute 1:14 with distilled water to obtain the M/15 solution used in preparing the medium.

Figure 11.
Rice Powder Dispenser


I CONSTRUCTION
II WRAPPING FOR STERILIZATION
100 mm. Petri Dish
4 Layers pf cheese cloth, holding rice powder
75 mm. Funnel
No. 4 Rubber stopper
25 mm. Glass tubing
Heavy paper tied with cord
Sterilize in autoclave at 121.6°C for 20 minutes
III USING THE DISPENSER

1. To charge dispenser, remove paper cover and Petri dish, add difco rice powder (previously sterilized with dry heat for 2½ hours at 150°C) and replace Petri dish and paper cover.
2. To use dispenser, slip off paper covering mouth, tap once or twice to deliver desired amount of powder, and replace paper cover.

All Procedures Should Be Performed Aseptically

Preparation of Medium

1. Mix well the yolks of 4 hardboiled eggs with 125 ml of 0.8% sodium chloride. (If possible, use a Waring Blendor.)
2. Heat the mixture in a covered double boiler for 20 minutes after it reaches 80°C. Add 20 ml distilled water to make up for evaporation.
3. Filter through a Büchner funnel under reduced pressure using several layers of #2 paper.
4. Autoclave the filtrate 20 minutes at 15 lbs.
5. Cool to below 10°C. Refilter through the Büchner funnel.
6. Add an equal amount of M/15 buffer solution to the filtrate to complete the yolk infusion.
7. Add 1 part of the liver extract solution to 9 parts of the yolk infusion.
8. Autoclave at 15 lbs (121°C) for 20 minutes.
9. The medium may either be stored or tubed immediately in 7 to 10 ml amounts.

LIVER INFUSION AGAR—This medium consists of a solid liver infusion agar slant with an overlay of physiological saline and fresh horse serum (Cleveland and Collier, 1930; Cleveland and Sanders, 1930). It is claimed to be more specific for *E. histolytica* than for the other protozoa which do not grow well on it.

Serum Saline Solution (Overlay)

Fresh horse serum	1 part
Physiological saline (0.8% NaCl in distilled water)	6 parts

Mix and sterilize by filtration.

Agar Solution

Dehydrated liver infusion agar (Difco)	30 gms
Disodium phosphate (Na_2HPO_4)	3 gms
Distilled water	1,000 ml

Dissolve the agar and disodium phosphate in the distilled water.

Agar Slants

1. Dispense the liquid agar into tubes in an amount sufficient to give an agar slant 4 to 5 cm long *without* a butt.
2. Sterilize at 15 lbs pressure for 20 minutes.
3. Add sufficient overlay to cover the slant.

COMMERCIAL MEDIUM—When only occasional cultures are desired, use the dehydrated base medium which can be purchased as Bacto Entamoeba Medium from the Digestive Ferments Co. (Difco), Detroit, Michigan 48201. Directions for preparation are on the bottle.

INOCULATION AND EXAMINATION TECHNIQUE—

1. Just before use, add approximately 30 mgm of sterile rice starch to each tube of medium. This may be done with a sterile wire loop (use 1 loopful) or a dispensing funnel (Figure 11) by 1 or 2 sharp but gentle taps on top.
2. For each specimen to be cultured, warm 2 tubes of medium to approximately 37°C.
3. To 1 tube, add enough penicillin and streptomycin to give a final concentration of 200 to 250 units of each per ml of overlay (Spingarn and Edelman, 1952; Norman and Brooke, 1955a). This is approximately 1,000 units of each antibiotic per tube.
4. Inoculate as follows:
   a) Liquid or semi-solid material.
   With a large bore pipette, add 0.5 ml of specimen to each tube of medium. Add as little air as possible. Mix the inoculum with the overlay, being careful not to introduce air bubbles.
   b) Formed feces.
   With applicator sticks, transfer a portion of specimen the size of a small pea to the tube of medium. Mix with the overlay by carefully rubbing the material against the side of the tube. Portions for inoculating are preferably selected from any mucus, blood, or abnormal appearing areas in the fecal material, if present.
5. Incubate all tubes at 37°C for 24 hours.
6. With a sterile, large bore pipette, place a drop or two of the sediment on a slide and cover with a coverslip. Prepare an unstained mount and a temporary stain preparation with Quensel's or buffered methylene blue solutions. The amebae grow at the bottom of the culture or on the surface of the slant near the bottom.
7. All cultures which show no trophozoites or an insufficient number for identification are transferred to fresh media. With a sterile large bore pipette, transfer about half of the sediment to a tube of medium. Do not add antibiotics to any of these transfers (secondary cultures).

8. Incubate both the original cultures and the transfers at 37°C for 48 hours and examine.
9. Sediment of cultures revealing organisms or questionable objects should be mixed in vials with PVA-fixative. Permanently stained films of the PVA-fixed sediments are prepared and carefully examined under the oil immersion objective (Norman and Brooke, 1955b).

SHIPPING MEDIUM—The media used for routine cultivation of protozoa in the laboratory are not suitable for transporting organisms from one location to another. Gleason *et al.* (1960) developed a special shipping medium in which trophozoites may survive for up to 4 days, thus permitting shipment of viable organisms to a central laboratory for recovery and identification. It is Balamuth's egg extract medium enriched with dextrose, trypticase, and sodium thioglycolate.

Modified Balamuth's Medium

Prepare as directed for Balamuth's Egg Yolk Infusion Medium, but omit the liver extract solution.

Enriched Locke's Solution

To 100 ml of modified Locke's Solution (formula on page 129), add the following:

Dextrose	110 mgm
Trypticase (BBL)	2 gms
Thioglycolate	50 mgms

Preparation of Medium

Balamuth's Medium (without liver)	5 parts
Enriched Locke's solution	1 part

Dispense in 6 ml volumes in screw-cap tubes (approximately 15×125 mm). Sterilize at 15 lbs pressure for 15 minutes. Cool and store in the refrigerator. If kept refrigerated, the medium may be used up to 3 months after preparation.

Method of Use

Emulsify a small amount of feces about the size of a small pea in a tube of medium in the usual manner for fecal cultures. Add approximately 1,000 units each of streptomycin and penicillin to the tube (250 units of each per ml of overlay). Screw the cap on tightly and ship in a double mailing container (or suitable equivalent) to the laboratory.

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Method of Use

Emulsify a small amount of feces about the size of a small pea in a tube of medium in the usual manner for fecal cultures. Add approximately 1,000 units each of streptomycin and penicillin to the tube (250 units of each per ml of overlay). Screw the cap on tightly and ship in a double mailing container (or suitable equivalent) to the laboratory.

When the specimen is received in the laboratory, replace the supernatant fluid with fresh medium (without adding antibiotics). Transfer about half of the sediment to a second tube of medium. If the amount of sediment is very small (as may be true with sigmoidoscopic specimens), do not transfer. Incubate tubes at 37°C and examine daily.

In positive specimens, motile organisms usually can be detected in 24 hours if the storage or transit time was not more than 1 day. Cultures kept at room temperature for longer periods may need to be incubated longer before growth is apparent. For example, cultures left at room temperature for 3 days, may not show motile organisms for 3 to 5 days at 37°C.

Although organisms can be recovered after 4 days at room temperature, best results will be obtained with 1- to 2-day old specimens.

EXAMINATION AND CULTIVATION OF ASPIRATES FROM LIVER ABSCESSES—Liver abscesses suspected of being caused by *E. histolytica* are often drained and the aspirate is examined in an attempt to determine the etiologic agent. Since demonstrating amebae in the thick pus removed from the abscesses is frequently difficult, proteolytic enzymes can be used in freeing the trophozoites from the aspirate material. The following procedure incorporates the recommendations of the Amoebiasis Research Unit, Durban, South Africa (Freedman, *et al.*, 1958; Elsdon-Dew, 1960).

Technique

1. During aspiration a minimum of 2 portions of exudate is removed. These portions are kept separate. The first portion withdrawn, usually yellowish-white, seldom contains amebae and is not examined routinely. Later portions, which are reddish, are more likely to include organisms. The final portion containing material from near the wall is most likely to be positive. (Collapse of the abscess and inflowing blood is believed to release amebae from the tissue.) Subsequent aspirations performed after appropriate rest periods of several hours have a greater chance of revealing organisms.
2. Ten units of the enzyme, Streptodornase, are added to each ml of thick pus and the mixture is incubated for 30 minutes at 37°C with repeated shaking. This process frees the amebae from the coagulum.

3. Centrifuge the mixture at 1,000 r.p.m. for 5 minutes. The sediments may be examined microscopically in wet mounts or used to inoculate culture media.
4. In culturing the material, the egg slant medium overlayed with Locke's solution is conditioned by inoculation with *Clostridium perfringens*. This can be done at the time the amebic pus is inoculated, though, if possible, the medium should be preconditioned for 24 hours at 37°C. Sterile rice powder is added when the aspirate is inoculated. Overlay with a mixture of paraffin and vaseline (1:1). Incubate the cultures at 37°C and examine for amebae after 24 and 48 hours.

HELMINTH LARVAE

In old fecal specimens both hookworm and *Strongyloides* rhabditiform larvae may be present. If doubt as to species exists, the feces may be cultured to obtain filariform larvae. Hookworm requires 5 to 6 days to reach the filariform stage; *Strongyloides* requires only 2 to 3 days. Two methods of cultivation are given: the charcoal sand culture and the test tube culture (Sasa *et al.*, 1958).

CHARCOAL OR SAND CULTURE

Preparation

1. Mix a large portion of feces (about the size of a walnut) with an approximately equal quantity of fine granular wood or bone charcoal or sterilized sand. Powdered charcoal is not satisfactory.
2. Using 2 tongue depressors, "cut" the feces into the sand to form small "lumps" about the size of small peas or smaller.
3. Add water to moisten the material, but avoid getting it too wet.
4. Place the mixture on moistened filter paper in a petri dish. Waxed half-pint cartons, such as those recommended for specimen collection, can also be used. The feces mixture tends to dry more rapidly in the carton, however, and daily "watering" may be necessary. To do this, sprinkle the surface of the culture until it appears visibly moist. Do not get the culture too wet. Cultures, whether in petri dishes or cartons, must be kept moist because drying will kill the larvae.
5. Keep the cultures at room temperature for 3 to 7 days. If petri dishes are used, larvae may be found in the drops of moisture on the lid. The lid or the surface of the culture may be examined with a hand lens or dissecting microscope to determine if larvae

are present. Filariform larvae will migrate to the surface of the mixture or to the moisture drops.

The culture should be checked for larvae on the 3rd day. If the species in question is *Strongyloides*, filariform larvae will probably be seen. If no larvae are visible, the culture should be checked again on the 5th and 7th days.

6. Species should be positively identified by microscopical examination of the larvae.

Recovery of Larvae from Cultures

Collect larvae as follows:

1. If present in the drops of moisture on the petri dish lid, wash the drops into a beaker, tube, or clean petri dish.
2. Use a Baermann apparatus to separate larvae from the culture material. A simple Baermann can be constructed from a large glass funnel with a short length of rubber tubing, fitted with a pinch clamp, attached to the bottom. A piece of wire screen is fitted into the top of the funnel and a layer of muslin (a piece of bed sheet is excellent) is placed over this (Figure 12). Gauze does not work as well as muslin, since it permits charcoal or sand to escape into the lower levels of water. The funnel is held upright by putting it into a ring stand.
    a) Fill the funnel with warm tap water (37°C).
    b) Place the top layers of the culture material, or the entire culture, if it is small, on the muslin-screen. The water should cover the bottom part of the material.
    c) Let stand for 30 minutes to 1 hour to allow the larvae to collect in the rubber tube and the lower part of the funnel.
    d) Draw off a few ml of the water into a beaker. If the larvae are few, the material can be centrifuged for 2 to 3 minutes at 1,500 r.p.m.
    e) Transfer a drop of the material (use the sediment, if centrifuged) to a slide and examine with the 10X objective.

TEST-TUBE CULTURE

1. Cut a strip of filter paper about 1 inch wide and 7 inches long.
2. Smear about ½ gm of feces (a quantity about the size of a small pea) over approximately ½ to ⅔ the length of the filter paper.

3. Place the strip in a large test tube wtih approximately 3 ml of tap water in the bottom. The lower edge of the filter paper should be in the water, but the feces does not have to be submerged.
4. Cover the tube—a cork stopper or cotton plug is satisfactory—and keep at room temperature for 3 to 7 days. Filariform larvae will be found at the bottom of the tube.
5. Examine a drop after 3 days for *Strongyloides* larvae and after 5 to 7 days, for hookworm larvae.

This method is simpler and easier than the charcoal or sand culture.

EXAMINATION

Examine unstained wet mounts for the presence of motile larvae, adjusting the light for optimum visibility. Rapidly moving larvae are difficult to observe. To kill the organisms, a drop of iodine or formalin can be allowed to run under the coverslip. Hookworm and *Strongyloides* filariform larvae can be differentiated by the length of the esophagus and the appearance of the tip of the tail. When cultures are examined at the end of 3 days, rhabditiform larvae of hookworm (alone or with *Strongyloides* filariform larvae) may be found. These are often larger than those seen in fecal specimens, but their characteristics are the same. Be careful to distinguish between rhabditiform and filariform larvae to avoid erroneous diagnoses. If fecal samples have been contaminated with soil, free-living larvae may have to be differentiated from parasitic larvae. The method for doing this is as follows (Shorb, 1937):

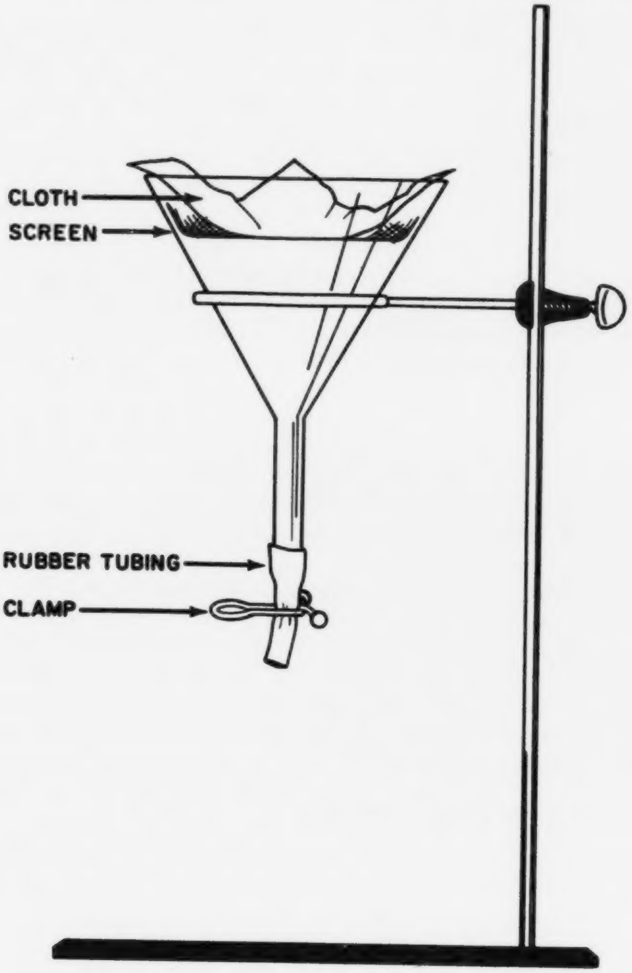
> Add 0.3 ml of concentrated hydrochloric acid per 10 ml of water containing larvae. A 1:30 concentration of hydrochloric acid is obtained. If less than 10 ml of suspension is available, use proportionate amounts of acid. Free-living nematodes are killed almost immediately; infective larvae of parasitic species live for about 24 hours. Larvae can be specifically identified by morphologic features.

RECOVERY OF LARVAE FROM SOIL

Soil samples are sometimes examined for the presence of larvae. These samples can be cultured in the same fashion as feces and the larvae recovered with the Baermann apparatus. In some cases, culturing may not be necessary, and the soil sample can be placed directly in the Baermann. Larvae may migrate out of the soil more slowly than from the feces culture, and recovery may require several hours.

Larvae of free-living species must be separated from those of parasitic species. (The method for doing this is described in the preceding section.)

Figure 12.
Baermann Apparatus for Recovery of Larvae from Cultures or Soil Samples



MISCELLANEOUS PROCEDURES

TECHNIQUES FOR DIAGNOSIS OF ENTEROBIASIS

Since the eggs of *E. vermicularis* are usually deposited outside the body on the perianal area rather than within the intestinal tract, anal swabs are used for diagnosing enterobiasis. Two types of swabs, the cellulose-tape slide preparation (Graham, 1941) and the vaseline-paraffin swab (Markey, 1950) are most commonly used for routine purposes. The procedure given below for handling cellulose-tape slides incorporates the modifications of Brooke *et al.* (1949) and Beaver (1949b). The technique for vaseline-paraffin swabs is that used at NCDC; it is based on the original Markey (1950) method.

CELLULOSE-TAPE SLIDE PREPARATION

Preparation

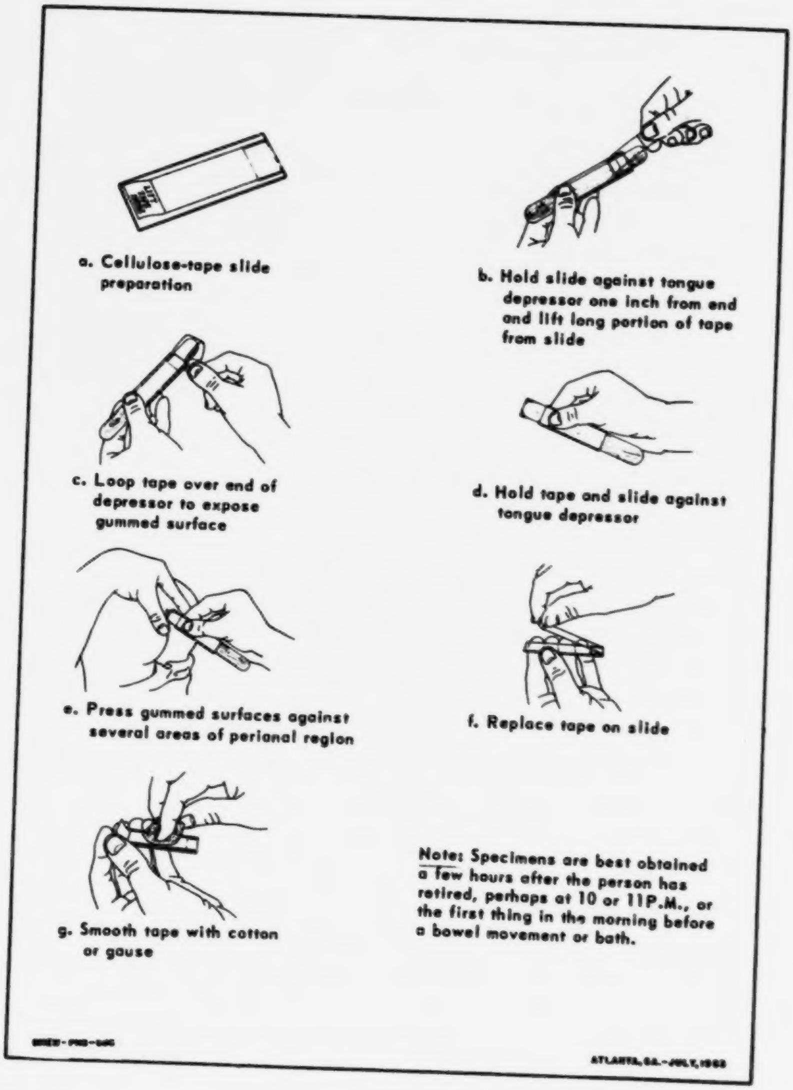
Clear cellulose tape, ¾-inch wide, is best for this purpose. Cloudy or hazy tape interferes with examination. A piece of tape about 4 to 5 inches long is anchored on the underside of a 3×1-inch slide and smoothed across the top of the slide. A paper tab is affixed to the free end of the tape for labeling and identification. (See Figure 13).

The unused tape preparations may be stored for several months in the refrigerator or in a cool place.

Technique

1. Collection (See Figure 13)
   - a. Pull tape back from slide, leaving the anchoring portion on the underside undisturbed, and, with sticky side out, loop over a tongue depressor or test tube to hold it steady.
   - b. Press the sticky tape surface against the perianal skin. The perianal folds should be pressed open to insure access to eggs concealed in the crevices. To facilitate examination, use only the center portion of the tape, if possible.

Figure 13.
Use of Cellulose-Tape Slide Preparation for Diagnosis of Pinworm Collections



a. Cellulose-tape slide preparation

b. Hold slide against tongue depressor one inch from end and lift long portion of tape from slide

c. Loop tape over end of depressor to expose gummed surface

d. Hold tape and slide against tongue depressor

e. Press gummed surfaces against several areas of perianal region

f. Replace tape on slide

g. Smooth tape with cotton or gause

Note: Specimens are best obtained a few hours after the person has retired, perhaps at 10 or 11P.M., or the first thing in the morning before a bowel movement or bath.

[illegible]

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ADAPTED FROM BROOKE, DONALDSON AND MITCHELL, 1949

   c. Smooth the tape back into place with a piece of gauze or cotton. Be careful not to contaminate hands.

2. Examination

   a. Lift the tape from the slide and place a drop of toluene or xylene on the slide. Smooth the tape back into position.
   b. Examine the center portion under the low power of the microscope. A 5X or 6X objective will facilitate observations.
   c. If eggs are not found in the center area, the rest of the slide should be scanned before reporting NPF (no parasites found).
   d. If the preparations are to be stored before examination, the toluene should not be added until the time of examination, since prolonged contact with the toluene may cause the organism to degenerate more rapidly than it normally would.
   e. Tape preparations may be stored in the refrigerator for several days or weeks before examination without extensive degeneration of the eggs.

VASELINE-PARAFFIN SWAB TECHNIQUE

Preparation

1. Dip a cotton swab into a mixture of 4 parts of vaseline to 1 part of parawax (paraffin) so that the swab is thoroughly coated. Let cool. If the mixture is too hot, the swab may have to be dipped twice to coat it thoroughly.
2. Put coated swab in a 100×13 mm tube and plug the tube with cotton. These swabs may be stored for long periods if the temperature is not so great that the vaseline-paraffin on the swab melts. Refrigeration is preferable.

Technique

1. Collection

   a. Rub the swab gently over the perianal surface, spreading open the perianal folds. Insert the swab about one-fourth inch into the anal opening.
   b. Replace swab in tube.

2. Examination

   a. Fill the tube about one-third to one-half full of xylene, just covering the swab, and let stand for approximately 3 to 5 minutes.

b. Remove the swab and centrifuge for 1 minute at 2,000 r.p.m.

c. With a pipette, carefully remove the supernatant; transfer the sediment to a 3×2-inch slide and examine for eggs. A coverslip is not necessary. To prevent the drop from spreading over a large area, draw a ring on the slide with a wax pencil. Some of the wax will dissolve, but the amount is usually not enough to interfere with the examination. A depression slide may also be used, but it necessitates more up and down focussing on different levels than the flat slide.

ESTIMATING WORM BURDENS

Egg counting techniques are used to evaluate the degree or intensity of infection. These procedures are chiefly used to estimate worm burdens in a population group in relation to control or eradication programs or to determine the efficacy of anthelmintics. The methods do not give the exact number of worms present, but they do furnish an approximation that enables one to classify the infections as light, moderate, or heavy.

The procedures can also be used with individuals, but the results must be interpreted with caution, since counts are not reliable in determining an individual patient's worm burden. Many factors, such as egg output of individual worms, influence individual counts. Counts may be of use, however, to a physician in classifying the general level of a patient's infection and in evaluating the effectiveness of a drug regimen by obtaining counts before and after treatment.

Several methods are available for making egg counts. The two commonly used for fecal specimens are the dilution egg counting technique (Stoll, 1923) and the standard smear technique (Beaver, 1949a, 1950). For estimating pinworm burdens, the coefficient of infection procedure is suggested (Sadun *et al.*, 1956).

DILUTION EGG COUNTING—This is a dilution method for determining the number of eggs per gram of feces. Since weighing out a specific number of grams of feces is very time consuming, the technique has been modified to obtain the specimen volumetrically by displacing an easily measurable quantity of liquid with feces.

This tends to yield counts approximately 5% too high, since 1 ml of soft feces has been found to weigh 1.03 to 1.04 grams, whereas the same amount of formed feces will weigh 1.05 to 1.07 grams. Such counts should be recorded as "eggs per ml" rather than "eggs per gram." These counts are sufficiently accurate, however, for routine purposes.

In some respects, the technique is similar to a blood count in that:

1. A measured quantity of feces is taken as the sample.
2. The sample is diluted in a constant volume of fluid.
3. A measured sample of homogeneous suspension is examined.

Decinormal sodium hydroxide is used as a diluent because it:

1. Saponifies fats.
2. Sets free eggs embedded in debris.
3. Makes the suspension clearer than water does.

Apparatus:

Stoll dilution flask—graduated at 56 ml and 60 ml* (Figure 14)
Stoll pipette (with rubber bulb)—graduated at 0.075 ml and 0.15 ml
Small glass beads (6 mm)
#4 rubber stopper
22×40 mm coverglasses (No. 2)
3×2-inch slides

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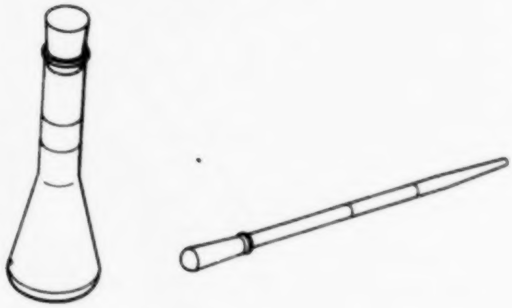
*Stoll flasks and pipettes are available from Arthur H. Thomas Co., Philadelphia.

Figure 14.
Stoll Flask and Pipette



Preparation of Solutions:

Sodium hydroxide	4 gms
Distilled water to make	1,000 ml

Dissolve NaOH in a small amount of water in 1-liter volumetric flask, then add additional distilled water to make 1,000 ml. The approximate normality is 0.1N. The solution does not need to be standardized for egg counting purposes.

Procedure:

1. Fill the special Stoll flask with 0.1N sodium hydroxide to the bottom mark etched on the neck of the flask (56 ml).
2. Using two applicators, add sufficient feces to the fluid to raise the level to the upper mark etched on the neck (60 ml).
3. Add 6 to 8 glass beads and firmly insert a No. 4 rubber stopper.
4. If the feces is very hard, allow it to soak for a while.
5. Shake vigorously with a straight up and down motion for 1 minute to secure a homogeneous suspension. Some workers recommend shaking the flask in an inverted position. A circular motion must be avoided, since this motion may concentrate the eggs in a small area, defeating the purpose of the shaking.
6. Routinely, it is best to run the sample to this point in the afternoon and then set it aside until the next morning. In so doing, a noticeable additional amount of eggs will be liberated from the fecal debris. Reshake tne contents of the flask the following day. Complete and even mixing is essential for an accurate result.
7. The eggs and debris will begin to settle out immediately after shaking stops. Using a Stoll pipette, *immediately* and quickly withdraw a sample of either 0.075 or 0.15 ml from the center of the suspension. The latter size is recommended since it provides a larger sample for counting. Inserting the tip of the pipette to the center of the flask decreases the error in counting due to settling of the eggs.
8. Expel the entire contents of the pipette on to a 3×2-inch slide.
9. Cover the drop with a 22×40 mm, No. 2 coverslip. Do not seal, since the paraffin may obscure some eggs.
10. Examine the mount systematically with the low power objective of the microscope. Cover the entire coverslip area.
11. Count all of the hookworm eggs present in the preparation, taking care not to count the same egg twice.

12. Multiply the number obtained by 100 if a 0.15 ml sample was used, or by 200 if a 0.075 ml sample was used. This will yield the total number of eggs per ml of formed feces. With mushy stools, multiply this latter number by the correction factor 2, with diarrheic stools, by 4.

Calculations on which step 12 is based (with 0.15 ml sample):

A. 4 ml of feces to 56 ml of 0.1N NaOH =
   4 ml of feces in 60 ml of suspension OR 1 ml of feces in 15 ml of suspension.

   Therefore, in the 0.15 ml of suspension counted, 0.01 ml of feces is present, and the number of eggs counted should be multiplied by 100 to give the eggs per ml or eggs per gram.

B. Obviously a given volume of formed stool will contain more fecal material and eggs than an equal volume of liquid or diarrheic stool, which essentially represents a diluted specimen.

   In a gram of formed stool, approximately 44 eggs have been found to represent one female *Necator americanus* worm. In a mushy stool, however, the egg count per gram has generally been found to be 22 and in a diarrheic stool, 12. This yields a ratio of 1:2:4 (formed:mushy:diarrheic). It is best to convert all counts to a "formed stool" basis by multiplying the count obtained from mushy stools by 2, and from liquid stools by 4.

Interpretation of Results:

Egg counts are applicable chiefly to infections with nematodes such as *Ascaris*, hookworm, and *Trichuris*, where the eggs are passed more or less continuously. Quantitative egg outputs have been used primarily in estimating hookworm burdens, particularly *N. americanus* burdens, and the procedure was originally developed for use in determining densities of infections with this worm in population groups. The interpretation of results, therefore, usually relates to hookworm, but it could be applied to other infections in which the egg output per gram of feces is known.

The number of *N. americanus* worms necessary to produce clinical symptoms varies from person to person and depends on several factors. As stated, a female *Necator* will yield an average of 44 eggs per gram of formed feces. Thus, a technique which can determine the number of eggs in a gram of feces in a given sample enables one to calculate the number of female worms present. Since males and females are usually found in almost equal numbers, doubling the original calculation yields the approximate total worm infection. Various workers consider that when there is a burden of 100 or more hookworms, clinical symptoms are likely to occur. This intensity level is represented by 2,600 or more eggs per ml of feces and is considered a moderate infection. In their hookworm surveys, Keller *et al.* (1934, 1940) used the following classification:

Intensity Grouping *(Eggs per ml of feces)*	Clinical Classification
100 - 699	Very light
700 - 2,599	Light
2,600 -12,599	Moderate
12,600-25,099	Heavy
25,100 and over	Very heavy

For routine purposes, it is usually sufficient to indicate the clinical classification based on eggs per ml of feces. The number of worms present need not be calculated.

STANDARD SMEAR EGG COUNT—The direct smear technique for egg counting involves using a light meter or photo-electric foot-candle meter in preparing a fecal smear of a known standard density.

Apparatus (Figure 15)

1. Light meter. The meter used should have the window on the same side as the scale.
2. A lamp, adjustable in height, suspended directly over the window of the meter. Either a 75- or 100-watt bulb may be used.
3. Block about 15 mm thick, 2 to 3 inches long, and with a central hole 16 mm in diameter. The inner surface of the hole may be covered with black ink to reduce light reflection and interference. The block is placed over the meter window to reduce the opening and to provide a uniform light area.

The apparatus can be standardized or calibrated by using barium sulfate suspensions as standards.

Preparation of Solutions

Stock Solutions

Solutions of 2N sodium sulfate (Na_2SO_4) and 1N barium chloride ($BaCl_2$) are prepared by analytical methods.

1. Na_2SO_4 (anhydrous) .... 142.05 gms
   Distilled water to make .... 1,000.0 ml

   In a 1-liter volumetric flask, dissolve the Na_2SO_4 in a part of the water, then add water to the 1 liter level.
2. $BaCl_2 \cdot 2H_2O$ .... 122.16 gms
   Distilled water to make .... 1,000.0 ml

   Prepare as for Na_2SO_4 solution.
3. Prepare salt-glycerin mixtures by mixing (separately) 2 parts of each salt solution with 1 part of pure gylcerin. The salt-glycerin mixtures are stored separately and mixed in varying proportions to give the desired concentrations standard.

Standards

The barium chloride solution is added to the sodium sulfate solution slowly, drop by drop, with constant stirring. The proportions used for various standards are as follows:

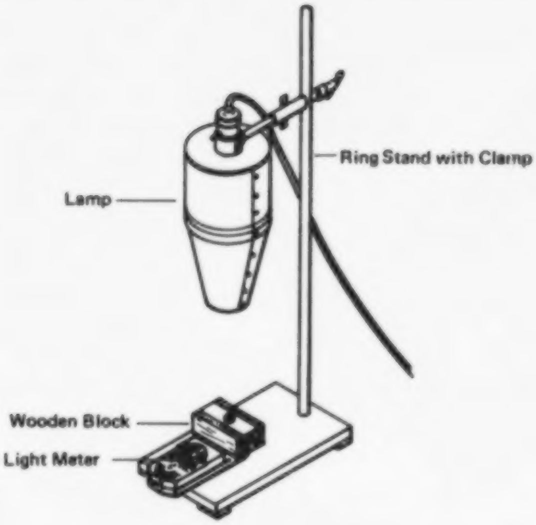
Parts barium chloride mixture	Parts sodium sulfate mixture	Give barium sulfate suspension for standardizing smears containing feces in the amount of:
3	2	1/200 ml
2	3	1/300 ml
1	2	1/400 ml
1	3	1/500 ml (2 mgm)
1	6	1/1,000 ml (1 mgm)

Calibration of Light Meter

1. Adjust the light to a predetermined reading on the scale (Beaver used 20 foot-candles as a setting).
2. Place a clean 3×1-inch slide over the opening of the block covering the light meter window.
3. Using a pipette which delivers 0.05 ml per drop (a regular medicine dropper is usually satisfactory), place a drop of the *thoroughly mixed* barium sulfate suspension in the desired concentration, on the slide over the block opening. The 1/1,000 (1 mgm) or 1/500 (2 mgm) standards are preferred for routine purposes.
4. With an applicator stick, carefully spread the suspension to exactly fit the opening. If the standard extends beyond this area, the slide should be discarded and a new one prepared.

5. Read the scale. Subsequent fecal preparations should be of sufficient density to cause the same light reduction.
6. With some meter scales, it may be easier to adjust the light so that a known, easily read scale reading is the end point. The main considerations are to determine the reduction in light intensity with the desired standard concentration and to prepare fecal mounts of the same density.
7. After adjusting the light and calibrating the apparatus, the position should not be changed. Periodic checkings are necessary, however, to be sure that current fluctuations or jarring have not affected the adjustments. In some areas, a voltage regulator is desirable.

Figure 15.
Lamp and Light Meter Apparatus for Standard Smear Egg Count



Preparation of the Fecal Smear

A 1 mgm or 2 mgm fecal suspension is usually easier to examine than thicker mounts, and the light meter should be calibrated with the corresponding standard (1/1,000 or 1/500).

1. Using a pipette which delivers 0.05 ml drop (medicine dropper), place a drop of distilled water on a clean slide directly over the block opening.

2. With an applicator stick, emulsify feces in the water, spreading the film to fit the block opening *exactly*, until the reading on the scale is the same as that of the corresponding standard. If the preparation extends beyond this area or too much feces is added, it should be discarded and a new slide prepared.
3. Cover with a 22 mm-square coverslip and, beginning with one corner, systematically count the number of eggs in the preparation. Do not seal the mount, since the paraffin may obscure some eggs.
4. Use of a 5X- ocular will decrease the examination time. Be careful not to overlap fields and recount eggs already counted.
5. To obtain eggs-per-gram count, multiply the number of eggs counted by 1,000 if a 1 mg preparation is used or by 500 if a 2 mgm preparation is used. No correction factors for soft or loose stools, as required by the Stoll count, are necessary. If no eggs are found in the 2 mgm preparation, report "less than 500 eggs per gram." A 3 mgm (or heavier) preparation is too thick to count the eggs accurately.
6. If several counts are to be made, the meter should be checked with the standard at intervals to make sure the readings have not altered. This is especially important if the flow of electric current fluctuates. A voltage regulator may be used if the current fluctuation is pronounced.

Interpretation of Results

Egg counts are interpreted in the same manner as with the Stoll dilution count. In both cases, the counts are estimates and provide an *estimate* of the existing worm burden rather than a specific count.

Use of Non-Standardized Smear

If no light meter is available, a rough estimate of the worm burden can be made from ordinary direct wet mounts prepared for routine examination. Experienced technicians usually prepare smears containing 1.5 to 2 mgm of feces. For practical purposes, the number of eggs per gram can be estimated by using 750 for a multiplication factor in step 5. Some laboratories report simply "eggs per preparation" without attempting to convert the count to eggs per gram. This is satisfactory so long as the person receiving the information can interpret the results. For example, 1 or 2 eggs per smear may represent a very light infection while 15 or 20 may represent a heavy infection.

COEFFICIENT OF INFECTION FOR ENTEROBIUS INFECTIONS—The number of *Enterobius* eggs on a cellulose tape preparation does not reflect the number of worms the patient harbors, since many variables, such as time and method of collection, hygienic habits of the patient, and the egg-laying habits of the female worm, may affect the number of eggs picked up by the tape. Egg deposition apparently does not occur every day, and thus it can be assumed that the greater the number of worms present, the greater the chances that eggs will be found on the day of collection. With 6 consecutive daily collections, if all 6 swabs are positive, the worm burden can be assumed to be higher than if only 1 or 2 positives are found.

On the basis of these assumptions, a relative measure of the average intensity of infection can be determined by calculating the coefficient of infection (CI) (Sadun *et al.*, 1956) as follows:

1. Cellulose tape swabs are collected daily for 6 days from the patients concerned. The swabs should be collected at about the same time each day, preferably between 10 and 12 p.m. The perianal folds should be spread so that the sticky surface of the tape picks up eggs that are present.
2. Examine the tape preparations in the usual manner. Clearing the debris with toluene will facilitate the examination. (See page 146).
3. Record whether positive or negative. Only the presence or absence of eggs is significant; the number of eggs is not.
4. Calculate the CI of a group of patients by adding the products obtained by multiplying the percentage of individuals having positive specimens on no occasion, once, twice and so on up to six times by the corresponding factors, 0, 1, 2, 3, 4, 5, or 6.

Example: In a group of 100 patients
30% are positive 0 times = 0
20% are positive 1 time = 20
10% are positive 2 times = 20
20% are positive 3 times = 60
5% are positive 4 times = 20
10% are positive 5 times = 50
5% are positive 6 times = 30
CI = 200

5. For individual patients, the intensity of infection can be determined by examining 6 consecutive daily swabs and assigning a value of 1 to a patient found positive 1 time out of the 6 daily

specimens, a value of 2 to a patient positive on 2 occasions, and so on up to 6. A patient with a CI of 6 would be positive with every examination.

Interpretation

It can be assumed that the higher the value of the CI, the heavier the infections. For example, if a group has a CI value of 200, the worm burdens can be assumed to be heavier than a group with a CI of 100, and lighter than a group with a value of 360. Likewise, an individual patient with a value of 6 has a heavier infection than a patient with a value of 1 or 2. Although the CI is a rough estimation of worm burden, it can be useful for clinical purposes and for evaluating the effect of therapy.

EGG HATCHING FOR SCHISTOSOME MIRACIDIA

When feces containing mature living schistosome eggs are diluted with water, the eggs hatch within a few hours. The miracidia tend to collect in the upper layers of the water where they may be located with a magnifying glass such as a hand lens. This is an especially sensitive test for indirectly demonstrating small numbers of *viable* eggs present in a fecal sample. McMullen and Beaver (1945) recommended a flask with a side arm, but for routine purposes an Erlenmeyer flask may be substituted.

TECHNIQUE

1. Place a sample of feces about the size of a large walnut (approximately 5 gms) into a 250 ml beaker and fill it one-fourth full with water. It is best to use chlorine-free water throughout this entire procedure, since tap water may contain a chlorine content sufficient to kill miracidia. Chlorine-free water may be obtained by boiling tap water for several minutes. The water should then be cooled to room temperature.
2. Comminute *thoroughly* using a tongue depressor.
3. Add sufficient water to fill the beaker three-fourths full.
4. Strain this suspension through 2 layers of wet cheesecloth into a sedimentation glass or cone-shaped graduate.
5. Allow the suspension to settle for 1 hour.
6. Carefully pour off supernatant fluid without allowing sediment to escape.
7. Resuspend the sediment in a small quantity of water and pour it into a 500 ml Erlenmeyer flask.

8. Fill the flask almost to the lip with water.
9. Allow the flask to stand at room temperature for several hours or overnight.
10. The eggs will hatch, liberating the miracidia, which will swim to the upper layers and collect in the neck of the flask. Thus, the organisms are concentrated in a small area and are more easily visible.

    Caution: If the water is cloudy, the miracidia may be found in all layers of the fluid. This cloudiness may be prevented by washing the sediment once more after Step 6.
11. Against a dark background, the miracidia will be observed as minute, white organisms swimming rapidly in a straight course. They tend to collect around the margin of the flask (Chernin and Dunavan, 1962). A hand lens will aid in detecting the larvae.
12. Coprozoic, free living ciliates, for example, *Colpoda steini*, may quickly appear in fecal-contaminated water and should not be confused with miracidia. To confirm identification, transfer a few organisms to a 3×2-inch slide and examine under the microscope. A drop of a weak iodine solution or dilute methylene blue will stop the motion of the miracidia.

PROCEDURE FOR MATURING COCCIDIA OÖCYSTS

Immature oöcysts of *Eimeria* species and of *Isospora* species are similar in appearance and may be difficult to distinguish. Man occasionally ingests oöcysts of *Eimeria* accidentally or with animal tissue and subsequently passes them in the feces. Therefore, generic identification of oöcysts found in a stool specimen may be important. Differentiation is based on the number of sporocysts present in the mature form and the number of sporozoites within each sporocyst.

Mature oöcysts can be obtained by mixing the feces with saline or water, placing a small quantity in a petri dish, and allowing it to stand at room temperature for 2 to 3 days.

Better results will be obtained if bacterial growth is retarded by mixing the feces with 2% potassium dichromate solution as follows:

PREPARATION OF POTASSIUM DICHROMATE SOLUTION

Potassium dichromate ($K_2Cr_2O_7$) crystals	2 gms
Distilled water	100 ml

Dissolve the crystals in the water. Store in a glass stoppered bottle.

TECHNIQUE

1. Emulsify a portion of the feces in about 2 volumes of potassium dichromate solution.
2. Pour a small amount into a petri dish to a depth of about one-fourth inch.
3. Cover and leave at room temperature for 2 to 4 days. Be sure the suspension does not dry up. Add a small amount of distilled water if necessary.
4. With a pipette, remove a drop of the sediment to a 3x2-inch slide and cover with a coverglass. Examine under low and high power for the presence of mature oöcysts. Stains, such as iodine, are not necessary. The potassium dichromate, however, may stain the oöcysts slightly.
5. If the oöcysts are too few to be detected in this manner, the material can be concentrated by centrifugation. Transfer the entire contents of the petri dish to a 15 ml conical centrifuge tube and centrifuge at 2,000 r.p.m. for 1 to 2 minutes. Examine preparations from the sediment as described above. If the sediment is too dry or too thick, a drop of saline should be used in making the mount.

EXAMINATION OF RECTAL BIOPSY TISSUE

Rectal biopsies have been used with success in the diagnosis of schistosomiasis mansoni (Ottolina and Atencio, 1943) and amebiasis (Juniper *et al.*, 1958).

SCHISTOMIASIS

Generally, a small piece of tissue is removed from the level of the dorsal fold (Houston valve) with tracheal forceps. Ottolina (1951) demonstrated that the greatest concentration of eggs occurs at about 9 cm from the anus; thus the dorsal valve is a convenient biopsy area. No special preparation of the patient is necessary before biopsy. However, the bowel should be free of feces.

Technique for Examining

1. Rinse the tissue in saline to remove blood and debris.
2. Press between 2 slides and examine with the low power magnification of the microscope.
3. Living mature eggs appear somewhat transparent, amber-colored, and with a well-defined miracidium. Immature eggs, which are filled with a greyish granular mass, and calcified, dead eggs, which appear black, may also be seen (Maldonado, 1967).

4. Examination of the biopsied tissue may be facilitated by adding a drop or two of lacto-phenol (1:1) and letting the tissue stand a few minutes. This procedure clears the tissue, and the eggs may be more distinct than in untreated tissue.
5. Sectioning and staining biopsied tissue is less satisfactory than examining pressed tissue for eggs and is not recommended for diagnosis of *S. mansoni* infections.

AMEBIASIS

Tissue is obtained by a punch biopsy technique. Juniper *et al.* (1958) recommended using only lesions located below the middle valve of Houston. Minute mucosal lesions may be removed *in toto*, or if lesions vary in size, specimens should be obtained from both small and large ulcerations. Ulcers selected for biopsy should not be scraped. Prompt fixation and good histologic technique are necessary for positive identification of trophozoites.

Technique for Examining

1. Remove the biopsied material from the forceps and place on a piece of 3×5-inch card with the mucosal surface down. The specimen should be kept flat. Lift out edges that may curl under with toothpicks. All fragments of mucus and exudate should be retained with the tissue.
2. Immediately place the card with the specimen face down in 10% formalin for fixation.
3. Prepare a tissue block in the manner normally used for histologic specimens, being careful to include any tissue fragments or exudate.
4. Section and stain by the routine histologic procedures using hematoxylin-eosin stain prepared as follows:
   a) Stock Hematoxylin Stain
      (1) Solution 1

Hematoxylin	5 gm
Absolute ethyl alcohol	50 ml

Dissolve the stain in the alcohol.

(2) Solution 2

Aluminum ammonium sulfate	100 gm
Distilled water	1,000 ml
Ferric chloride	3 gm

Dissolve the ammonium alum in the water with the aid of heat. Add the ferric chloride.

(3) Mix solutions 1 and 2 and bring to a boil.

(4) Add 2.5 gm of mercuric oxide. The solution will turn dark purple.

(5) Remove the solution from the heat and cool in cold water.

b) Stain solution for use:

Hematoxylin stock solution	180.0 ml
Distilled water	50.0 ml
Glacial acetic acid	2.4 ml

Add hematoxylin stock solution to the distilled water and mix. Add the acetic acid and mix again.

TECHNIQUES FOR TRICHINOSIS

While trichinosis is more frequently diagnosed by serologic methods, it is occasionally desirable to examine biopsied tissue for the presence of larvae. In addition, diaphragms or other tissue obtained at human autopsy or from pigs may be examined. Several techniques are available, but the compression and digestion techniques are reliable (within the limits of tissue examination) and simple to perform.

COMPRESSION TECHNIQUE

This is a simple and rapid method of demonstrating trichina larvae in tissue. Light infections may be missed, however, unless a large portion of tissue is examined. Such an examination would require several preparations. The compression technique is valuable in detecting long-standing infections in which the larvae are dead or calcified and in early infections in which the larvae are immature. In addition, no special equipment is needed. Gould (1945) advocated a simple slide compression as a routine procedure and a more elaborate method using a ring-shaped trichinoscope. The simple slide method is described here.

Technique

1. Cut very thin slices from the tissue specimen and press between 2 slides.
2. Examine with low power for the presence of larvae.
3. A drop of eosin may be added to the tissue to slightly stain the larvae.

DIGESTION TECHNIQUE

The digestion technique permits examination of a larger tissue sample than compression or sectioning and staining methods and may result in the detection of light cases that might otherwise be missed. It recovers living larvae very well, but it does not recover dead ones. Furthermore, the digestion technique may destroy immature larvae and thus miss early infections. Also, digestion will cause calcified cysts, usually present in long standing infections, to disintegrate. Nolan and Bozicevich (1938) suggested the following procedure.

Equipment

1. Erlenmeyer flask—100 or 150 ml size. Larger sizes will be needed if large samples of tissue, such as whole diaphragms from pigs or human autopsies, are to be handled.
2. Mechanical shaker or stirrer.
3. Baermann apparatus. For small tissue samples of approximately 1 gram, only a small funnel is needed. Larger samples will require a proportionately larger apparatus.
    a) To the neck of a small funnel (2 to 3 inches wide) attach a short 2- to 3-inch length of rubber tubing fitted with a pinch clamp.
    b) Place a piece of fine screen, about 80 mesh, in the top of the funnel. If a regular sieve with a metal edge is not available, use a circle of fine screen wire and turn the edges up about ½-inch or so to make it fit down inside the funnel.
    c) Put the funnel apparatus in a ring holder on a ringstand to hold it upright.
4. Slides—3×2-inch size.
5. Pipette with rubber bulb.

Preparation of Solution

Artificial Digestive Juice

Pepsin	5 gms
Hydrochloric acid, concentrated	7 ml
Distilled water	1,000 ml

Dissolve the pepsin in the water and add the acid. Mix thoroughly.

Technique

1. Place 1 gm of finely ground tissue in 20 ml of digestive juice in a small Erlenmeyer flask.
2. Place flask in a 37°C incubator for 18 hours or longer.
3. Stir mixture thoroughly at half-hour intervals.
4. Add 2 to 3 volumes of water heated to 37° to 45°C to the digestate. The exact amount is unimportant.
5. Pour the diluted digestate into the Baermann funnel and add enough warm water to cover the screen.
6. Let stand 1 hour or longer. The larvae will collect in the lower part of the tube and in the neck of the funnel.
7. Draw off a few ml of fluid and place a drop on a 3×2-inch slide. Examine with the 10X objective for living larvae.
8. If no larvae are found, the sample can be centrifuged and the sediment examined.
9. Large tissue specimens will require larger quantities of digestive fluid. For a sample of 50 grams or so, approximately 3 liters will be needed for proper digestion.

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